VOLUME 31 JUNE 1953 NUMBER 3

Canadian Journal of Medical Sciences

Editor: J. B. COLLIP

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

CANADIAN JOURNAL OF MEDICAL SCIENCES

(*Formerly Section E, Canadian Journal of Research*)

The CANADIAN JOURNAL OF MEDICAL SCIENCES is published bimonthly by the National Research Council of Canada under the authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research. Matters of general policy are the responsibility of a joint Editorial Board consisting of members of the National Research Council of Canada and the Royal Society of Canada.

The National Research Council of Canada publishes also: *Canadian Journal of Botany, Canadian Journal of Chemistry, Canadian Journal of Physics, Canadian Journal of Technology, Canadian Journal of Zoology.*

Manuscripts should be addressed to:

Dr. Léo Marion,
Editor-in-Chief,
Canadian Journal of Medical Sciences,
National Research Council,
Ottawa, Canada.

Each manuscript should be typewritten, double-spaced, and the original and one extra copy submitted (see **Notes to Contributors** inside of back cover).

Subscriptions, renewals, and orders for back numbers should be addressed to:

Administrative Services,
National Research Council,
Ottawa, Canada.

Subscription rate: $3.00 a year; single numbers: 50 cents. Special rates can be obtained for subscriptions to more than one of the Journals published by the National Research Council.

Canadian
Journal of Medical Sciences

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 31 JUNE 1953 NUMBER 3

VARIATION OF THE HYDROGEN ION CONCENTRATION OF HEALTHY INTACT SKIN[1]

BY W. R. INCH[2] AND ALAN C. BURTON

Abstract

Beckman glass and calomel electrodes were used in conjunction with a Beckman model G pH meter to determine the hydrogen ion concentration of the skin surface. Three types of experiment, carried out on male and female subjects, were designed primarily to study the degree of normal variability of the skin reaction and the related E.M.F., in view of measurements being made on the potentials of tumors. The experiments were (*a*) 20 min. studies, (*b*) 26 hr. studies and (*c*) 48 day studies. The short term studies showed that in all cases the skin pH readings reached an equilibrium value after two to four minutes and remained quite steady to the end of the experiment. The diurnal variation of the skin reaction in all subjects followed a cyclic path which exhibited excellent reciprocal correlation with environmental temperature. Subjects kept in a constant temperature, constant humidity environment did not show this cyclic pattern in their readings. Oral administration of atropine excluded the possibility of sweat gland activity influencing the temperature dependence. Long term studies on both male and female subjects showed wide variation in absolute readings and little or no evidence of a cyclic nature could be observed or statistically shown.

Introduction

Although investigators had previously measured the pH of exuded sweat from the human body surface (11), quantitative determinations of the hydrogen ion concentration of the 'intact skin' were not reported until 1923 by Sharlit and Scheer (10). These investigators used the available methods of that time, which were mainly colorimetric, by direct application of indicators to the skin, and arrived at values of the correct order (based on present day measurements), but which contained large errors due to improper color matching. The first report of electrometric methods being used to measure skin pH was by Schade and Marchionini in 1928 (9) and Levin and Silvers in 1932 (5) who used the hydrogen and quinhydrone electrodes respectively. These methods were both subject to errors brought about by the presence of oxidizing agents, reducing agents, proteins, and other electrode poisons.

[1] *Manuscript received January 15, 1953.*

Contribution from the Department of Biophysics, Medical School, University of Western Ontario, London, Ontario. This work was supported by a research grant from the National Cancer Institute of Canada.

[2] *Holder of a Fellowship from the National Cancer Institute of Canada.*

In 1906 Cremer (2) investigated the changes in potential which occurred across a glass membrane separating solutions of different hydrogen ion concentrations. Haber and Klemensiewicz (4) realized the possibility of using such glass in the construction of electrodes sensitive to the hydrogen ion and did some experimental work to this end, but not until much later was it carried out to the extent of constructing working electrodes which were both rugged and flexible (3, 6). In 1938 Blank employed this newly developed glass electrode to measure the skin pH of human subjects and reported in a series of papers (1) the values that he obtained on different areas of the healthy skin surface of adults and children. He found that the normal skin surface of adults (19 to 27 years) exhibited a pH, as measured by the glass electrode, between 4.0 and 7.0 with most readings between 4.2 and 5.6, and that there was some indication that a monthly cycle existed in women. There was little correlation between pH and environmental temperature. Also he found that for the male the average pH for children was more alkaline than for the adult, but that, in the female, there was little or no age difference.

The following investigation covers part of the work of a larger study of the d-c. bioelectric potentials exhibited by normal and pathological tissue. Tumor tissue often has a negative potential with respect to neighboring normal tissue, and a different pH. The study of the fluctuations of pH of normal tissue is a necessary preliminary to the study of pH of skin over tumors.

Apparatus and Procedure

Three types of experiments were carried out on male and female subjects, designed primarily to study the degree of normal variability of the skin reaction. The experiments were (*a*) 20 min. (short term) studies, (*b*) 26 hr. (diurnal) studies, both in a varying temperature environment and in an environment of constant temperature and humidity, (*c*) 48 day (long term) studies.

A Beckman model G pH meter in conjunction with Beckman calomel and glass electrodes was used for all skin reaction measurements. The instrument was modified by the insertion of longer electrode leads and the addition of shielding to the leads and electrodes (see Fig. 1*b*). The electrodes were spring mounted in slots cut in the shielding material so that the distance and angle between them could be adjusted slightly (see Fig. 1*a*). This instrument was accurate to 0.02 pH units and readings could be reproduced to within 0.05 pH units. Since no experimental value had a pH of more than 8.0 there was no sodium or potassium error in the readings and no correction was applied (7, 8). The meter was calibrated before each set of determinations using buffers of pH = 4.0 and pH = 7.0. Measurements were carried out on the flexor surfaces of both arms 10 cm. craniad from the most distal skin crease at the wrist. This site was chosen because of the thinness of the overlying skin, the absence of hair, and convenience both for subject and investigator during the determination.

PLATE I

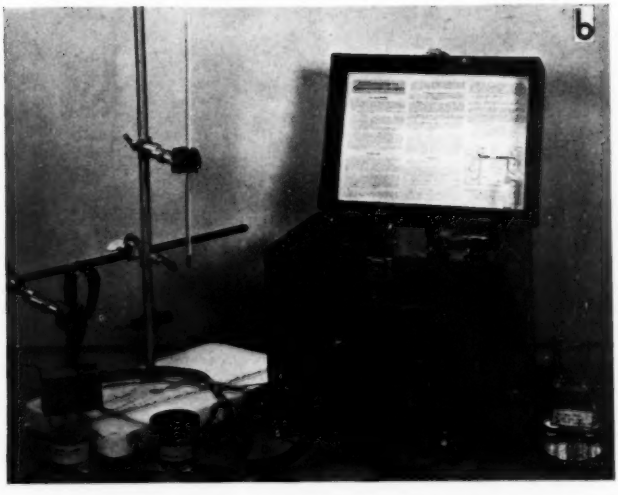


FIG. 1. (*a*) Glass and calomel electrodes mounted in shielding material.
(*b*) Modified Beckman pH meter used for skin measurements.

The technique used in carrying out individual pH measurements was a modified version of that used by Blank. The skin surface at the point of measurement was prepared by first washing with ethyl ether and then moistening two small areas (approx. 1 cm. in diameter and 1 cm. apart) with 0.1% sodium chloride solution previously adjusted to a pH of 6.5 with dilute hydrochloric acid (1). The electrodes were applied to these areas and moved slightly in a circular path, and more solution was added to ensure good contact. Readings were taken at the end of two and four minutes, after the electrodes were first applied to the skin surface. The latter reading was considered to be the true skin pH as explained later.

In all, 12 male or female subjects between the ages of 24 and 40, with no apparent skin lesions or previous history of skin ailments, were used in the experiments. They were instructed not to wash or apply any substance to the portion of the skin upon which measurements were to be made within 10 hr. of the experiment. This was necessary because the application of soap (as well as many creams, lotions, and ointments) to the skin surface renders it extremely basic for many hours even though it is rinsed with clean water and prepared as above. In addition, subjects were instructed not to indulge in strenuous exercise or take stimulants of any kind within an hour of the measurements.

Observations

(*a*) The short term studies were carried out in order to determine the optimum time at which the pH reading should be taken. This was a compromise between several factors (1) the slope of the pH–time curve, (2) the practical time which could be allowed for the measurement, and (3) the reproducibility of the reading.

The data for a typical experiment is plotted on Fig. 2. The fluctuation, after the first four minutes, is less than 0.1 pH unit. It was concluded from

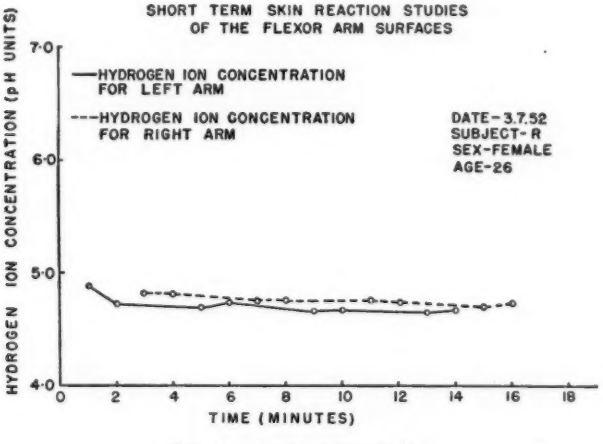


FIG. 2. Short term studies.

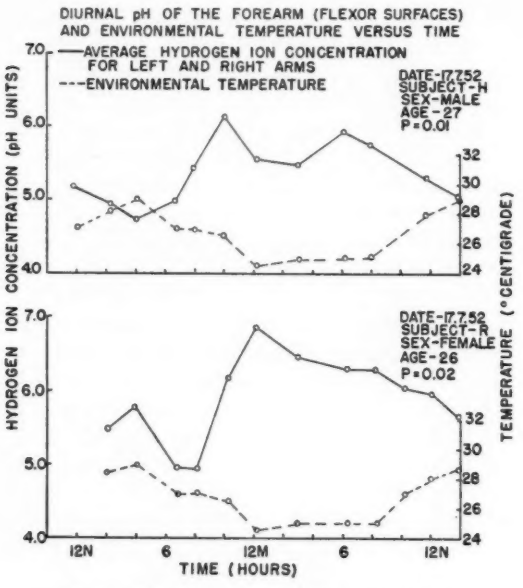


FIG. 3. Diurnal studies, varying temperature.

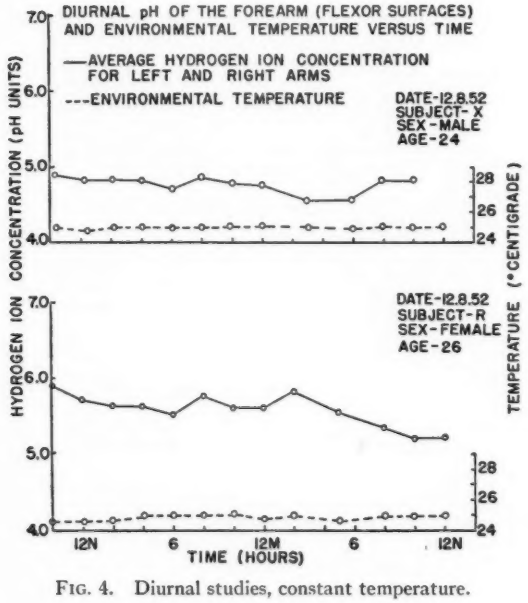


FIG. 4. Diurnal studies, constant temperature.

the examination of many similar curves that readings should be taken at the end of two and four minutes and, providing that these did not differ by more than 0.1 pH units, the latter reading would be accepted as the true skin pH reading. In the cases where the readings differed by more than 0.1 pH units, which were not frequent, the data were noted but not accepted and the skin was washed and prepared again. This procedure stabilized the reading in all cases. No correction was applied to the final reading for environmental temperature fluctuation, since this was considered negligible in the 20 min. period of measurement, when the temperature did not change by more than one-quarter degree Centigrade.

(*b*) The diurnal pH studies were divided into two groups (1) those carried out in a varying temperature environment with no control on humidity, (2) those carried out in a constant temperature, constant humidity environment. As nearly as possible, the subjects followed their normal routine of activity and sleep. Data from both types of experiment are plotted on Figs. 3 and 4.

Fig. 3 reveals that there is a definite inverse relation of the skin pH with the environmental temperature. It can be seen that the pH reaches a low point (corresponding to high acidity) in the late afternoon, lagging slightly behind the environmental temperature maximum, rises sharply to a high point (corresponding to low acidity) at about midnight (when the temperature is least), falls gradually during the night and more rapidly during the morning until it returns almost to the original value at the end of 24 hr. Statistical analysis of the data from seven experiments using five subjects indicated that there was an excellent correlation between the skin pH and environmental temperature, Table I. In all experiments, values of P between 0.01 and 0.06 were obtained.

In view of the relation between pH of skin and the environmental temperature, the second group of experiments on diurnal variation were made at constant temperature (23° C.) and humidity (50%). Fig. 4 shows the results in two cases and it is evident that the skin pH remains relatively constant when the environmental temperature is constant. An apparent trend to decrease

TABLE I

COEFFICIENTS OF CORRELATION BETWEEN SKIN pH AND ENVIRONMENTAL TEMPERATURE IN THE DIURNAL STUDIES

Subject	Coeff. corr.	Standard error, r	P
R	−0.56	±0.20	0.02
R	−0.54	±0.19	0.02
X	−0.58	±0.19	0.02
X	−0.54	±0.20	0.02
W	−0.49	±0.22	0.06
E	−0.60	±0.18	0.01
H	−0.63	±0.18	0.01

of pH is perhaps indicated in one of the subjects. The case cited is the only one which occurred in four experiments carried out in the constant temperature room. In any event smaller fluctuations than were seen in the other experiments show no diurnal rhythm.

(*c*) Long term studies were carried out on four male and four female subjects. All readings were taken at approximately the same time each day and the data obtained from two such studies (one male and one female) are shown in Fig. 5. From these it is observed that the skin pH varies markedly from day to day, although the values for each arm usually agreed within 0.1 pH units and there was no obvious cyclic pattern. The environmental temperature (in this case outside temperature, since laboratory was not heated) also exhibited fluctuations but no correlation could be obtained between pH and temperature. Statistical analysis of the data yielded *P* values between 0.1 and 0.4.

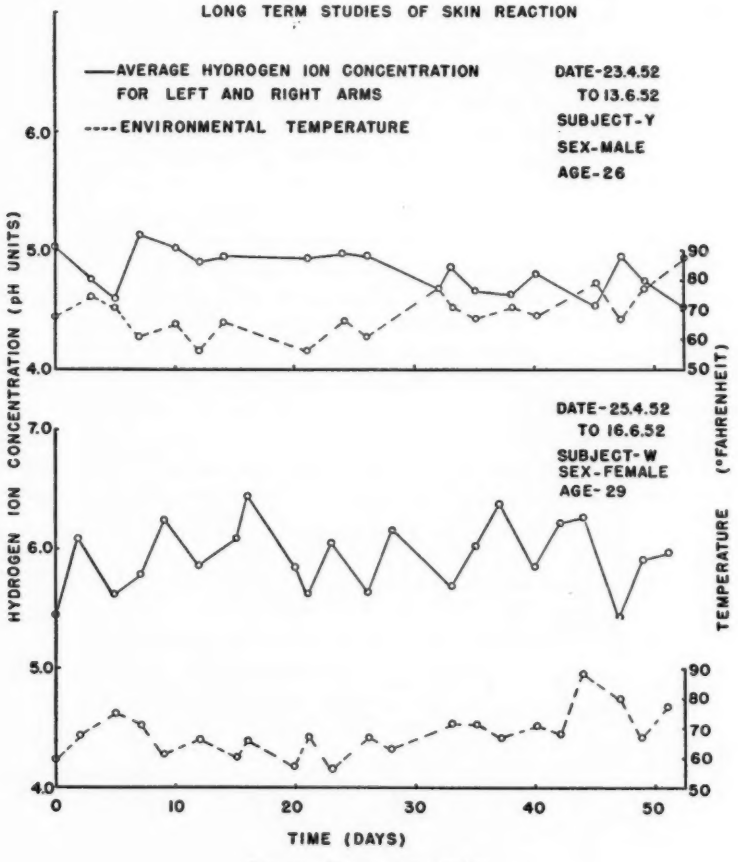


FIG. 5. Long term studies.

(*d*) The close relation between fluctuations in pH of the skin and the environmental temperature, seen in the diurnal experiments, suggests that this might be due to the activity of the sweat glands, as influenced by the temperature. Some experiments therefore were made after oral administration of atropine (1/100 to 1/60 grain in a single dose) to inhibit sweat gland activity. The activity of the glands was checked by the use of the method described by Thaysen, Schwartz, and Dole (12), making use of iodine sublimed on to bond paper. In the three subjects used, the sweating was very markedly inhibited after atropine, on the area used for the pH studies. The results were different on different subjects. In two subjects the dependence upon temperature was still marked and similar to that previously obtained on the same subjects without atropine (Fig. 6). In one subject however, the dependence upon temperature seemed to be greatly diminished after atropine.

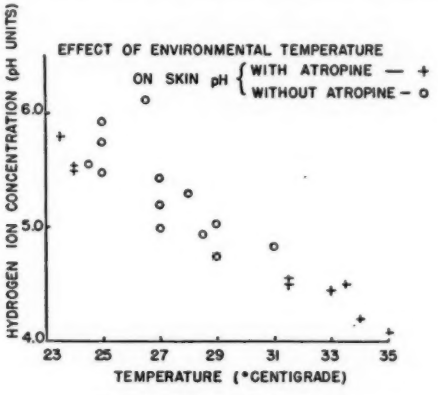


FIG. 6. Effect of atropine.

Discussion

There is considerable fluctuation of the pH of the skin of a given subject, on a given region of the body, throughout the day and from day to day. In the long term studies for example, the pH of the skin of the forearm in one subject averaged 5.93 with a S.D. of $\pm$ 0.29. This fluctuation is very greatly reduced for the same subject kept at constant temperature for 26 hr. when the mean was 5.77 with a S.D. of only $\pm$ 0.11. There is certainly no 'diurnal' rhythmic fluctuation when the temperature is kept constant. We would conclude that a change of more than 0.5 pH units in the reaction of the skin, when temperature conditions are constant, would be significant as in measurements on tumors, where differences in pH from normal as great as 1.5 pH units have been measured.

The nature of the dependence of the pH of the skin upon external temperature is not clear. The obvious theory that it might be due entirely to changes in sweat secretion is not borne out by the fact that a similar dependence on temperature can be shown in subjects after atropine has demonstrably

inhibited sweat-gland activity in the area concerned. Also, sweat is said to be more alkaline than skin (11), so that an increase in glandular activity, as with increased environmental temperature, would be expected to raise the pH. The actual change of pH of the skin with increased temperature is the opposite, i.e. pH falls when temperature increases. Also in the preparation of the skin surface, sweat should be removed and play no part in the results, unless the sweating were very great. The change in the metabolic rate and perhaps in carbon dioxide of the skin with increased skin temperature might be responsible.

Conclusions

(1) In order to obtain the pH of the skin surface with any degree of reproducibility, readings should be taken only after the system has reached an equilibrium value. Using the apparatus as described above, this point can be determined by two (or more) pH readings taken after the electrodes have been in contact with the skin surface for at least two minutes. Of the readings taken, the latter one is considered the true skin pH if it does not differ from the reading taken two minutes previously by more than 0.1 pH units.

(2) The diurnal variation in the hydrogen ion concentration of the normal human skin surface follows a cyclic pattern inversely related to the environmental temperature. In the experiments where the environmental temperature was held at a constant value the skin pH was also constant within the limits of the measuring technique which again tends to corroborate the dependence of the skin pH on environmental temperature. However, after inhibition of the sweat gland activity in the region of skin concerned by atropine, the dependence on environmental temperature was still present. Further considerations also indicate that the dependence on environmental temperature is not due to sweat gland activity.

(3) The normal skin reaction varies considerably from day to day and this fluctuation cannot be correlated with any degree of significance to environmental temperature. For a given area of skin in a given subject a change in pH of 0.5 units can be considered as significant. In view of this, the changes in pH from normal skin which have been measured in tumors are significant.

References

1. Blank, I. H. J. Investigative Dermatol. 2 : 67, 75, 231, and 235. 1939.
2. Cremer, M. Z. Biol. 47 : 562. 1906.
3. Dole, M. The glass electrode. John Wiley & Sons, Inc., New York. 1941.
4. Haber, F. and Klemensiewicz, Z. Z. physik. Chem. 67 : 385. 1909.
5. Levin, O. L. and Silvers, S. H. Arch. Dermatol. and Syphilol. 25 : 825. 1932.
6. MacInnes, D. A. and Dole, M. Ind. Eng. Chem. Anal. Ed. 1 : 57. 1929.
7. Perley, G. A. Private correspondence.
8. Perley, G. A. Anal. Chem. 21 : 559. 1949.
9. Schade, H. and Marchionini, A. Arch. Dermatol. u. Syphilis, 154 : 690. 1928.
10. Sharlit, Herman and Scheer, Max. Arch. Dermatol. and Syphilol. 7 : 592. 1923.
11. Talbert, G. A. Am. J. Physiol. 50 : 433. 1919.
12. Thaysen, J. H., Schwartz, I. L., and Dole, V. P. Federation Proc. 1, 11 : 161. 1952.

SOME EFFECTS OF VITAMIN B_{12} IN WEANLING RATS CONSUMING HYPOLIPOTROPIC DIETS[1]

By C. H. Best, C. C. Lucas, Jean M. Patterson, and Jessie H. Ridout

Abstract

Kidney lesions may develop and death ensue in very young rats consuming hypolipotropic diets in the presence of vitamin B_{12} even when subprotective amounts of choline are available unless the diet contains sufficient methionine. The necessity is established for a careful adjustment of the dietary concentrations of both choline and methionine before the protective effect of vitamin B_{12} on kidney lesions may be elicited.

Study of the interrelationships among antianemia factors, biological methylating agents, and growth factors for rats, chicks, and bacteria culminated in the isolation of a common factor, vitamin B_{12} (14, 22). The finding that an unidentified factor essential for rat growth occurs in products such as milk, commercial casein, liver extracts, muscle, and egg yolk, but is absent from yeast, corn meal, wheat flour, and soybean meal (5, 11), seemed unrelated to the observation that sardine fishmeal contains an unknown factor which exerts a sparing action on the dietary requirement (of chicks) for methionine (13) or to the report (4) that a factor in cow manure spares the methionine requirement of chicks. These and many other seemingly unconnected observations provided clues suggesting that the "animal protein factor" is concerned in some way with reactions involving labile methyl groups.

Prominent among these other observations were the findings of the workers at the Lankenau Hospital Research Institute. In 1943 and 1944 they reported (1, 25) that, in contrast to the general impression, their rats would grow on a synthetic diet containing homocystine but no methionine or choline.

Subsequent studies related the findings to the dietary history of the rats. Several excellent reviews are available dealing with the interrelationships among the growth factors, the lipotropic factors, vitamin B_{12}, and the biological synthesis of labile methyl groups (6, 7, 12, 26, 27).

The suggestion indicated above (1, 4, 13, 25) of a partial interchangeability of choline, methionine, and vitamin B_{12} was confirmed by the report of Schaefer *et al.* (19, 20) that vitamin B_{12} decreases the incidence and severity of the hemorrhagic kidney lesions which result in weanling rats from a diet low in choline and methionine (8, 9). It was found that a certain minimal (subprotective) amount of choline was necessary in the basal diet before any protective effect of vitamin B_{12} could be elicited (20).

The experiments to be described were undertaken because this laboratory has been interested in attempts to assess the relative lipotropic potencies of the several compounds exhibiting this property (3). It soon became apparent

[1] *Manuscript received January 23, 1953.*

Contribution from the Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada. The expenses of this investigation were defrayed in part by grants from the Banting Research Foundation and from the Nutrition Foundation.

that the protective effect of vitamin B_{12} on the hemorrhagic kidney lesions of weanling rats could not always be demonstrated even when the subprotective amount of choline was present. This led to a study of the conditions under which vitamin B_{12} provides some protection. Because methionine may serve as a source of labile methyl groups for the biosynthesis of choline, it is desirable in lipotropic studies to keep dietary methionine low. A basal diet had therefore been adopted that was considerably lower in methionine than others had used. It appeared that the variability of response might depend upon the methionine content of the basal diet and this was confirmed in the experiments described below.

Methods

White rats of the Wistar strain, raised in our own colony, were used. Usually the animals were placed for a few days before the test period in individual metal cages which possessed a false bottom of coarse wire screen, in order to accustom them to the environment. The animals within each group were chosen by random. The dietary ingredients were mixed in a Hobart power food mixer and the rations were stored in tightly covered tinned cans in a refrigerator at about 4° C. Weighed amounts of fresh diet were given daily in galvanized iron feed trays designed to minimize spilling. The following morning the amounts of food left over and scattered were weighed; from these weights individual daily food consumptions were calculated.

When paired-feeding was adopted, this was done on a group basis, that is, each rat in the test group received the average amount of food consumed the previous day by the rats of the control group which were eating ad libitum. Animals were weighed at least twice per week and in some experiments daily. Fresh water was available at all times. Rats that died during the experiments were autopsied. At the end of the experiment the surviving animals were stunned and the livers were removed immediately, wiped free from blood, and weighed. The extraction of liver lipids with hot alcohol and analysis of the petroleum ether-soluble liver fat have been described elsewhere (2). The kidneys were examined in the gross for lesions; in doubtful cases a histological study was made.

Proteins from vegetable sources (peanuts and soybeans), which are known to be low in vitamin B_{12}, were used exclusively in all diets. The protein components of the diets have been analyzed by microbiological methods* for choline and essential amino acids. The choline content of the alcohol-extracted peanut meal was so low that the value could not be determined very accurately but it was of the order of 0.001%. The soybean protein (Glidden's 'alpha protein') contained less than 0.005% of choline. The extracted peanut meal as used contained about 0.57% methionine and the alpha protein about 1.05%. For purposes of calculation values of 0.6 and 1.0%, respectively, were used. The composition of the experimental diets is given in Table I.

* *We are indebted to our colleague, Miss Jessie M. Lang, for these analyses.*

Dose–Response Studies with Vitamin B_{12} in Weanling Rats

As is shown in Table II the effects of vitamin B_{12} alone (Expt. I), of choline chloride alone (Expt. II), of folic acid alone (Expt. III), and of various combinations of these (Expt. IV) were studied in weanling female rats. Male rats were used in the remaining studies. In Expt. V (Table III) the effects of choline chloride alone, of vitamin B_{12} in the presence of choline, and of vitamin B_{12} plus folic acid in the presence of choline were examined.

Since deterioration of vitamin B_{12} in the diet, or variable absorption from the gut, might affect the results, a comparison was made in Expt. VI of vitamin B_{12} given at equal dosage in the diet and by intraperitoneal injection in physiological saline. A control group (VI–0s) injected with saline alone and another group (VI–1s) getting dietary vitamin B_{12} was also injected intraperitoneally with saline. Vitamin B_{12} is known to exert a marked influence on the rate of growth, hence paired feeding experiments were conducted with

TABLE I

PERCENTAGE COMPOSITION OF BASAL HYPOLIPOTROPIC DIETS

Component	A	B	C	D	E
Peanut meal*	12	18	12	12	12
Soya protein**	12	12	12	12	12
L-Cystine	0.2	0.1	0.1	0.2	0.1
DL-Methionine	—	—	0.2	—	—
L-Lysine HCl	—	—	0.3	0.3	0.3
DL-Threonine	—	—	0.1	0.1	0.1
Salt mixture***	3	3	3	3	3
Lard	—	20	20	20	20
Beef fat	15	—	—	—	—
Corn oil	5	—	—	—	—
Sucrose	51.77	45.88	51.27	51.37	51.47
Sucrose – vitamin mixture†	1‡	1‡‡	1‡	1	1
Cod liver oil concentrate††	0.015	0.015	0.015	0.015	0.015
α-Tocopheryl acetate	0.010	—	0.010	0.010	0.010

* *Solvent-process peanut meal extracted with hot ethanol (50%, 70%, and 95%, respectively).*

** *Glidden's 'Alpha Protein'.*

*** *The salt mixture (Lucas & Patterson) is one used in this laboratory, made from salts commercially available in finely powdered form and supplying amounts of minerals believed optimal for growth of rats: 1 kgm. contains $CaCO_3$, 110 gm.; $CaHPO_4$, 325; K_2HPO_4, 275; $MgSO_4 . 3.5\ H_2O$, 100; NaCl, 150; ferric citrate, 30; "trace element mixture", 10 gm. The latter contains, per 100 gm.: $CaCO_3$, 70; $MnSO_4 . 4H_2O$, 19.85; $ZnSO_4 . 7H_2O$, 3.50; $CuSO_4 . 5H_2O$, 4.00; KI, 0.05; NaF, 0.05; $Al_2(SO_4)_3$. $K_2SO_4 . 24\ H_2O$, 0.40; $CoCl_2 . 6\ H_2O$, 0.05; $Na_2SiO_3 . 9H_2O$, 2.00; and $NaAsO_2$, 0.10.*

† *The 'sucrose-vitamin mixture' consisted of thiamine hydrochloride, 500 mgm.; riboflavin, 250 mgm.; pyridoxine hydrochloride, 200 mgm.; calcium pantothenate, 1.00 gm.; nicotinic acid, 1.00 gm.; folic acid, 50 mgm.; biotin, 30 mgm.; 2-methyl-1,4-naphthoquinone, 100 mgm.; p-aminobenzoic acid, 10 gm.; inositol, 50 gm.; and finely powdered sucrose (100 mesh), to 1000 gm.*

†† *Obtained from Ayerst, McKenna & Harrison, Ltd., Montreal; contains 200,000 I.U. vitamin A and 50,000 I.U. vitamin D per gm.*

‡ *Folic acid omitted from sucrose–vitamin mixture.*

‡‡ *Biotin omitted from sucrose–vitamin mixture.*

TABLE II

EFFECT OF VITAMIN B_{12} IN WEANLING FEMALE RATS

Diet A, supplemented as shown, fed ad libitum to animals weighing 36 to 42 gm., for 17 days

Expt. and group No.	Supplements per 100 gm.			Deaths	Incidence of kidney lesions	Mean values for survivors		
	Choline chloride, mgm.	B_{12}, μgm.	Folic acid, μgm.	Starters		Total liver lipids, % wet weight	Daily food consumption, gm./day	Change in weight, gm.
I – 0		0		17/20	19	29.5	3.7	+ 3
– 1		2		10/10*	10	*	4.6	
– 2		4		10/10*	10	*	5.7	
– 3		8		8/10	10	26.7	5.6	+14
II – 0	0			17/20	19	29.5	3.7	+ 3
– 1	20			9/20	18	23.6	4.4	+14
– 2	40			1/20	5	23.0	5.4	+22
– 3	60			0/10	0	14.3	5.5	+23
– 4	80			0/10	0	9.5	5.3	+22
III – 0			0	17/20	19	29.5	3.7	+ 3
– 1			200	8/10	9	24.2	5.4	+11
– 2			400	7/10	10	26.7	4.8	+ 1
– 3		4	200	8/10	10	28.7	5.6	+23
IV*a* – 0	20			9/20	18	23.6	4.4	+14
– 1	20	8		8/10	10	32.3	5.1	+32
– 2	20		400	8/10	9	29.4	4.7	+15
– 3	20	8	400	3/10	7	27.1	5.5	+32
IV*b* – 0	40			1/20	5	23.0	5.4	+22
– 1	40	2		3/10	9	23.7	4.7	+27
– 2	40	4		0/10	2	20.9	5.4	+33
– 3	40	8		2/10	6	27.2	5.3	+41
– 4	40		200	1/10	2	20.8	5.6	+14
– 5	40		400	1/10	4	19.5	5.8	+25
– 6	40	4	200	0/10	6	23.0	5.7	+35

* *One moribund survivor, killed on the 15th day, had liver lipids over 20%.*

TABLE III

Effect of vitamin B_{12} in weanling male rats

Rats weighing 38 to 44 gm. were fed for 17 days, except in Expt. VIII, in which older rats (40 to 50 gm.) were used and the duration was 14 days

Expt. and group No.	Basal diet	Supplements per 100 gm.			Deaths / Starters	Incidence of kidney lesions	Mean values for survivors			Fed	Remarks
		Choline chloride, mgm.	B_{12}, µgm.	Folic acid, µgm.			Change in weight, gm.	Daily food consumption,* gm.	Total liver lipids, ** % fresh wt.		
V-0	A	20			12/25	24	+13	4.9	24.9	Ad libitum	
-1		20	8		12/20	19	20	5.3	21.2	Ad libitum	
-2		20	16		17/20	20	21	5.8	(24.8)	Ad libitum	
-3		20	16		8/15	11	10	4.5	11.8	Paired with V-0	
-4		20	16	800	20/20	20	—	—	—	Ad libitum	
VI-0	A	20			9/10, 10/20	10, 20	+7, +1	4.0, 3.3	(26.3)	Ad libitum	
-1		20	8		9/10	10	23	4.7	(27.5)	Paired with VI-0	
-2		20		400	8/10	10	4	4.7	(15.8)	Ad libitum	
-3		20	8	400	8/10	9	16	4.7	(14.6)	Paired with VI-0	
-0s		20			5/10, 12/20	9, 20	14, 2	4.9, 3.5	27.4	Ad libitum	Saline intraperiton.
-1s		20	8		15/20	20	18	5.6	28.9	Ad libitum	Saline intraperiton.
-1p		20	(8)		10/20	17	28	5.7	23.2	Paired with VI-1s	Vit. B_{12} intraperiton.
-3s		20	8	400	18/20	20	18	5.0	(15.4)	Ad libitum	Saline intraperiton.
VII-0	B	20		***	14/15	15	+12	4.2	(22.7)	Ad libitum	
-1		20	8	***	10/15	15	31	5.2	23.7	Ad libitum	
-2		20		***	14/15	15	13	4.1	(30.3)	Ad libitum	Vitamin E 50 mgm.
-3		20	8	***	12/15	15	28	5.6	(23.4)	Ad libitum	Vitamin E 50 mgm.
-4		20		***	13/15	15	14	4.7	(23.1)	Ad libitum	Vit. E 50 + biotin 100 µgm.
-5		20	8	***	13/15	15	36	6.4	(26.7)	Ad libitum	Vit. E 50 + biotin 100 µgm.
VIII-0	C	30		200	15/24	24	+21	5.0	19.6	Ad libitum	
-1		30	8	200	5/24	15	45	5.9	14.8	Ad libitum	
-2		330		200	0/6	0	58	8.1	6.5	Ad libitum	
IX-0	C	30			13/15	15	+40	5.9	(17.0)	Ad libitum	
-1		30		50	14/15	15	30	5.2	(27.1)	Ad libitum	
-2		30	8		6/14	6	62	7.4	18.1	Ad libitum	
-3		30	8	50	9/15	12	59	7.0	17.9	Ad libitum	
-4		180			0/6	0	64	7.5	5.8	Ad libitum	
X-0	D	30		***	8/20	20	+10	3.6	25.2	Ad libitum	
-1		30	8	***	11/20	19	27	5.4	20.1	Ad libitum	
-2		180		***	0/4	0	35	5.9	6.0	Ad libitum	

* *Mean values for food consumption may, in some cases, be misleading; see text for discussion.*

** *Liver lipids have limited significance where only one, two, or three surviving rats, and some of those in a sickly state, are involved. Such values are given in parentheses.*

*** *50 µgm. folic acid present in each 100 gm. of basal diet.*

certain groups in Expt. VI to assess the effect on weight changes and on the condition of the liver and kidneys of increased food intake due to improved appetite.

Expt. VII was conducted to determine whether the amount of vitamin E or the presence of biotin in the diet influences the effect of vitamin B_{12} on kidney lesions or liver fat because Diet A differed in these respects from that adopted by Schaefer *et al.* (20, 21). The possibility was also considered that the beef fat or corn oil in Diet A might be affecting the result and lard was therefore substituted for them in Diet B. Further comparison suggested that deficiencies of essential amino acids in Diets A and B (Table I) might be responsible for our failure to obtain a lipotropic effect from vitamin B_{12}. In Expts. VIII and IX the choline chloride was increased from 0.02 to 0.03% and certain essential amino acids were added (Table I, Diet C) to make the diets more nearly adequate, according to the tentative estimates of the minimal requirements suggested by Rose (15, 16, 17). In Expt. X (Diet D) the supplementary methionine was omitted from Diet C.

In Experiment XI 0.03% choline chloride was added to Diet D and this basal ration was supplemented with vitamin B_{12} alone, with methionine alone, and with the two together, respectively. The rats were fed ad libitum.

In Experiment XII (Diet E) DL-homocystine (0.18%) was substituted in a stoichiometrically equivalent amount for the methionine (0.20%) added in Diet C. The homocystine was added to determine whether lack of a methionine precursor rather than inadequate synthesis of methyl groups might account for the failure of vitamin B_{12} to protect the kidneys in the earlier experiments. These rats also were fed ad libitum.

Results and Discussion

Experiments I, II, and III were conducted simultaneously, and one group of 20 rats served as controls for all three. The basal ration (Table I, Diet A) produced fatty livers in weanling rats within a few days and caused an almost 100% incidence of hemorrhagic kidneys. In female rats the mortality ranged from 85 to 100%, most of the deaths occurring usually on the eighth or ninth days. Male rats are more susceptible to choline deficiency and succumb one to two days earlier. It may be pertinent to mention that this hypolipotropic basal diet contains less than 0.7 mgm. of choline per 100 gm. of diet (by microbiological assay of components). The control rats ate about 4 gm. per day so that their choline intake would be only about 0.03 mgm. daily, an amount which is negligible in comparison with requirement of the order of 5 to 10 mgm. under the conditions prevailing. The diet is probably free from betaine because the procedures used in the preparation of the protein sources would eliminate it. The methionine content is only about 190 mgm. per 100 gm. diet. The cystine content is also low (about 200 mgm.) so that the amino acid sulphur content is barely 100 mgm. To increase this, supplementary cystine (0.2%) was included in the basal ration, raising the organic sulphur to approximately 150 mgm. per 100 gm. of diet.

Under the conditions of Expt. I, vitamin B_{12} had no effect whatever on the deposition of liver fat, incidence of kidney lesions, or survival of the rats. In contrast, a clear-cut effect of choline was obtained in Expt. II on liver lipids, on the incidence of kidney lesions, and on survival. About 20 mgm. of choline chloride per 100 gm. of food gives a 50% survival rate with about 90% incidence of kidney lesions. Doubling this amount of choline results in about 95% survival with 25% incidence of kidney lesions. While 0.040% of choline chloride gives a maximal rate of gain in weight, it has a negligible effect on liver lipids in weanling rats consuming this type of diet. The results of this dose–response study in female rats indicated that the diet containing 20 mgm. of choline chloride per 100 gm. should prove suitable for studying the effect of vitamin B_{12} on kidney lesions and that the diet containing 40 mgm. should be useful in studying the effect of the vitamin on liver lipids.

No beneficial effect from the presence of folic acid alone (Expt. III) or of folic acid with choline was apparent in the results of Expt. IV*a* or IV*b*. Vitamin B_{12} had a favorable effect upon growth in the presence of both levels of choline but there was no clear-cut benefit on the incidence of kidney lesions nor any significant improvement in the liver lipids of the survivors. Inclusion of folic acid with choline and vitamin B_{12} in this type of diet made no obvious difference to the growth, kidney lesions, or liver fat.

The mean daily food consumption records in Tables II and III could be very misleading unless two points are noted carefully, viz., that the values are *mean* values and that they apply only to the *survivors*. For example, the mean daily food consumption of *all* rats started on Diet A in Group VI–0 was 5.2 gm. on the first day and 4.0, 4.0, 5.2, 5.7, and 4.7 gm., respectively, on the five subsequent days. About this time one or two rats apparently began to feel ill and their food intake fell off while that of others increased. Within several days all rats were seriously ill. The average food consumptions on the 7th to 11th days were 4.1, 2.7, 2.1, 2.1, and 3.0 gm., respectively. By this time six rats were dead, three were moribund, and one was rapidly recovering. Its food intake increased from 1.0 gm. on the 8th day to 5.9 gm. on the 13th day. Thus, recording a mean value of 4.0 fails to give a true picture of what actually occurred. This criticism applies particularly to the data for those groups in which a large number of kidney lesions and deaths occurred. These values are included, however, to illustrate the increased rate of growth that results from relatively small increases in food intake that occur when vitamin B_{12} is added to the ration.

In Expt. V the inclusion of vitamin B_{12} at 8 or 16 μgm. per 100 gm. of diet had no beneficial effect on the incidence of kidney lesions or survival of weanling male rats even when the diet contained 20 mgm. choline chloride. There was a slight improvement in food consumption and gain in body weight but no lipotropic effect whatever was noted except in the rats of Group V–3 which were pair-fed with those of the basal group. These rats had a somewhat lower incidence of kidney lesions and a smaller gain in weight but the mortality

was about the same as in the basal group. The inclusion of folic acid (Group V–4) seemed to worsen the situation since all the animals so treated died with kidney lesions.

It is obvious that in Expt. VI vitamin B_{12} had no lipotropic effect in weanling male rats consuming basal Diet A even in the presence of 0.02% of choline chloride. There was no significant difference between the results obtained in the rats given vitamin B_{12} intraperitoneally and those receiving it in the diet (compare VI–1, VI–1s, and VI–1p). Addition of folic acid to this diet had no beneficial effect on growth or the occurrence of kidney lesions either in the presence or absence of vitamin B_{12}.

These results and certain other studies not reported in detail showed that there are strict limitations on the conditions under which the sparing effect of vitamin B_{12} may be elicited in lipotropic studies. The consistent failure to obtain a protective effect from supplements of vitamin B_{12} led us to consider reasons for the discrepancy between these findings and those reported by Schaefer *et al.* (19, 20, 21). If too much choline or methionine is present in the basal diet, no lesions occur. On the other hand, if the diet permits lesions of too severe a nature to develop, vitamin B_{12} is incapable of remedying the condition. What other factors are also important was not apparent at this stage. The possibilities that biotin or α-tocopherol are concerned, or that beef fat or corn oil contain interfering substances, were first considered. The results of Expt. VII showed that these differences in the diets were not responsible for the results.

The diets in which Schaefer *et al.* observed the protective effect of vitamin B_{12} on kidney lesions contained about 0.3% of methionine in the protein components of their diets (peanut meal plus casein) and 0.04% of supplementary choline chloride (20, 21). When vitamin B_{12} was tested in Toronto in a diet containing 0.04% of choline chloride (Expt. IV*b*), no protection whatever was apparent. This diet contained considerably less (only about 0.19%) methionine.

Supplementation of Diet A with lysine, threonine, and methionine (with reduction of the cystine supplement to equalize approximately the organic sulphur in Diets A and C) and replacement of the fats used in Diet A by 20% of lard brought the composition of the basal diet more in line with that used in Alabama. In Expt. VIII, in which slightly heavier male rats, comparable to those used by Schaefer *et al.*, were fed this improved ration (Diet C), vitamin B_{12} reduced the incidence of kidney lesions and the mortality. The liver lipids in the basal group ranged from 14 to 26% with a mean value of 19.6% $\pm$3.65 (S.D.); in rats getting vitamin B_{12}, the liver lipids varied from 9 to 23% with a mean value of 14.8% $\pm$ 4.68 (S.D.). By Student's "*t*" test, the difference between these means is only moderately significant, the probability of such a difference occurring by chance being 0.015. Vitamin B_{12} improved the consumption of food and doubled the gain in weight. It is interesting to note that increasing the choline content of the ration (total choline chloride

0.33%, which is almost certainly more than adequate under these dietary conditions) also occasioned a marked improvement in food consumption and in gain of weight.

When slightly lighter rats were fed a similar diet lacking folic acid (Expt. IX), some protective effect of vitamin B_{12} on the kidney was confirmed. Because the numbers of survivors in the control groups are small (two and one, respectively) it is difficult to say whether vitamin B_{12} has exerted any lipotropic effect upon the liver lipids; if any reduction has taken place it is certainly very limited. Again it was noted that inclusion of folic acid in a diet of this type has a negligible effect on the protective action of vitamin B_{12} on kidney lesions.

The importance of the age of the rat is exemplified by comparing the protection afforded by vitamin B_{12} in Experiments VIII and IX. The basal diets and supplements are the same except that the older rats in Experiment VIII were given slightly more folic acid and our data indicate that this is not a beneficial factor, on the contrary, in our studies it has often aggravated the renal lesions. In the older rats 8 μgm. of vitamin B_{12} per 100 gm. of ration reduced the mortality from 15 out of 24 in the basal group to 5 out of 24 (63% to 21%), whereas in the younger rats the reduction was considerably less: from 14 out of 15 to 9 out of 15 (93% to 60%).

Others have also observed that it is more difficult to demonstrate a protective effect of vitamin B_{12} in the very young rats consuming hypolipotropic diets than in slightly older ones (10, 18, 23).

The importance of the methionine content of the diet is illustrated in Expt. X. When supplementary methionine was omitted from Diet C, as in Diet D, vitamin B_{12} did not exhibit even the limited protective effect on the kidneys noted in Expts. VIII and IX.

Table IV presents further data showing the dependence of the protective action of vitamin B_{12} on the methionine content of the diet. The rats on the basal ration, Group XI–0, gained on the average barely 0.5 gm. per day for seven to nine days at which time an abrupt loss of weight occurred and all

TABLE IV

DEPENDENCE OF THE PROTECTION AFFORDED BY VITAMIN B_{12} ON THE LIPOTROPIC ADEQUACY OF THE BASAL DIET

Male rats, 38 to 44 gm., fed Diet D supplemented with 0.03% choline chloride and further supplemented as shown. Duration 17 days

Expt. and group No.	Supplements per 100 gm.		Deaths	Incidence of kidney lesions	Mean change in weight of survivors, gm.
	B_{12}, μgm.	Methionine, mgm.	Starters		
XI – 0	0	0	8/10	10	+8
– 1	8	0	10/10	10	
– 2	0	200	7/10	10	27
– 3	8	200	2/10	4	58

but two of the rats died. Inclusion of vitamin B_{12} (Group XI–1) hastened the demise of the rats. All but one were dead by the 10th day and it died on the 12th. When supplementary methionine was added to the diet, growth was promoted, but neither the death rate nor incidence of kidney lesions was altered. However, the further addition of vitamin B_{12} doubled the rate of growth and considerably reduced the mortality and the incidence and severity of kidney lesions. These findings are in agreement with the data reported by Strength, Schaefer, and Salmon (24).

The possibility was considered that an adequate synthesis of labile methyl groups occurred in the rat but that the supply of methyl acceptors for the synthesis of compounds possessing lipotropic activity was insufficient. The data in Table V fail to support this hypothesis.

The present study confirms the observations of others that vitamin B_{12} can, *under certain conditions*, protect the kidneys of weanling rats fed an otherwise hypolipotropic diet. Some reduction of liver lipids may also be obtained when the age of the rat and composition of the basal diet happen to be so established as to permit demonstration of the effect. However, the main purpose of this communication is to emphasize the limitations upon the experimental situations under which the choline-sparing effect of vitamin B_{12} becomes manifest. Few biochemical processes require such careful adjustment of conditions for their demonstration. Whether this means that the biosynthesis of labile methyl groups under the influence of vitamin B_{12} is a process of limited *quantitative* significance, or whether other factors not yet recognized play an important role in altering the rate of this reaction, or of transmethylation, is not known. Inclusion of homocystine did not improve the situation. One may conclude that the results cannot be attributed to lack of methyl acceptors in the basal diets used in these studies. It is still to be established whether in older rats an increased rate of synthesis of methyl groups, or decreased requirement for lipotropic substances, or alteration of both explain the greater ease of demonstration of a protective role of vitamin B_{12} in the slightly older animals.

TABLE V

EFFECTS OF VITAMIN B_{12} AND OF HOMOCYSTINE

Weanling male rats (38 to 44 gm.) fed Diet E, supplemented as shown, for 17 days

Expt. and group No.	Supplements per 100 gm.			Deaths / Starters	Incidence of kidney lesions	Survivors, mean change in weight, gm.
	DL-Homocystine, gm.	B_{12}, μgm.	Choline Cl, mgm.			
XII – 0	0	0	30	6/10	9	+6
– 1	0.18	0	30	9/10	9	6
– 2	0.18	8	30	8/10	9	37

References

1. BENNETT, M. A., MEDES, G., and TOENNIES, G. Growth, 8 : 59. 1944.
2. BEST, C. H., LUCAS, C. C., PATTERSON, J. M., and RIDOUT, J. H. Biochem. J. 40 : 368. 1946.
3. BEST, C. H., LUCAS, C. C., RIDOUT, J. H., and PATTERSON, J. M. J. Biol. Chem. 186 : 317. 1950.
4. BIRD, H. R., RUBIN, M. and GROSCHKE, A. C. J. Nutrition, 33 : 319. 1947.
5. CARY, C. A., HARTMAN, A. M., DRYDEN, L. P., and LIKELY, G. D. Federation Proc. 5 : 128. 1946.
6. DU VIGNEAUD, V. A trail of research in sulfur chemistry and metabolism. Cornell Univ. Press, Ithaca, N.Y. 1952.
7. DU VIGNEAUD, V., RESSLER, C., and RACHELE, J. R. Science, 112 : 267. 1950.
8. GRIFFITH, W. H. *In* Biological Symposia. Vol. 5. *Edited by* Howard B. Lewis, Jaques Cattell Press, Lancaster, Pa. 1941. p. 193.
9. GRIFFITH, W. H. and WADE, N. J. J. Biol. Chem. 131 : 567. 1939.
10. HALE, O. M. and SCHAEFER, A. E. Proc. Soc. Exptl. Biol. Med. 77 : 633. 1951.
11. HARTMAN, A. M. Federation Proc. 5 : 137. 1946.
12. JUKES, T. H. and STOKSTAD, E. L. R. *In* Vitamins and hormones. Vol. 9. *Edited by* R. S. Harris and K. V. Thimann, Academic Press, Inc., New York. 1951. p. 1.
13. PATTON, A. R., MARVEL, J. P., PETERING, H. G., and WADDELL, J. J. Nutrition, 31 : 485. 1946.
14. RICKES, E. L., BRINK, N. G., KONIUSZY, F. R., WOOD, T. R., and FOLKERS, K. Science, 107 : 396. 1948.
15. ROSE, W. C. Science, 86 : 298. 1937.
16. ROSE, W. C., OESTERLING, M. J., and WOMACK, M. J. Biol. Chem. 176 : 753. 1948.
17. ROSE, W. C. and WOMACK, M. J. Biol. Chem. 166 : 103. 1946.
18. SCHAEFER, A. E. and KNOWLES, J. L. Proc. Soc. Exptl. Biol. Med. 77 : 655. 1951.
19. SCHAEFER, A. E., SALMON, W. D., and STRENGTH, D. R. Federation Proc. 8 : 395. 1949.
20. SCHAEFER, A. E., SALMON, W. D., and STRENGTH, D. R. Proc. Soc. Exptl. Biol. Med. 71 : 193. 1949.
21. SCHAEFER, A. E., SALMON, W. D., STRENGTH, D. R., and COPELAND, D. H. J. Nutrition, 40 : 95. 1950.
22. SMITH, E. L. Nature, 161 : 638. 1948.
23. STEKOL, J. A. and WEISS, K. J. Biol. Chem. 186 : 343. 1950.
24. STRENGTH, D. R. SCHAEFER, A. E., and SALMON, W. D. J. Nutrition, 45 : 329. 1951.
25. TOENNIES, G., BENNETT, M. A., and MEDES, G. Growth, 7 : 251. 1943.
26. WOODS, R. Borden's Review of Nutrition Research, 12 : 11 and 23. 1951.
27. ZUCKER, T. F. and ZUCKER, L. M. *In* Vitamins and hormones. Vol. 8. *Edited by* R. S. Harris and K. V. Thimann, Academic Press, Inc., New York. 1950. p. 1.

ENZYMATIC CHANGES IN THE LIVER OF MICE FOLLOWING FEEDING OF THE INSECTICIDE ALDRIN (HEXACHLOROHEXAHYDRODIMETHANONAPHTHALENE)[1]

By E. Annau and H. Konst

Abstract

The ingestion of sublethal doses of the insecticide aldrin, $C_{12}H_8Cl_6$, induces in mice after a period of time a considerable enlargement of the liver. It was considered possible that some enzymatic changes in the liver of the intoxicated animal might be responsible for some features of the pathological process. Activities of the following four enzymes were investigated: acid and alkaline phosphatase, arginase, and xanthine oxidase. The results indicated that the activity of acid phosphatase and arginase remained unchanged during the entire feeding period, while the activity of xanthine oxidase and alkaline phosphatase appeared to be decreased.

Introduction

In a previous communication (1) we discussed some aspects concerning the increase in the weight of the liver of mice fed aldrin in sublethal concentrations. It was stated that the increase may be due to an hypertrophy as well as hyperplasia of the liver cells induced by a preferential protein assimilation. The present communication describes attempts to investigate the effect of aldrin feeding on various enzymes presumably connected with protein metabolism. Acid and alkaline phosphatase, arginase, and xanthine oxidase were examined.

Materials and Methods

Male and female albino mice, derived from the stock colony of this Institute and ranging in weight from 20 to 25 gm., were used for the experiments. All animals were fed a ground Master Fox Breeder Starter laboratory chow. Aldrin in the form of a concentrated corn oil solution was mixed in the food of the experimental animals at a rate of 50 to 100 p.p.m. as indicated in the tables. The addition of corn oil raised the fat content of the experimental diet by 1% above that of the controls, an amount shown to have no effect on the lipid content of the liver (1). Food and water were given ad libitum.

Mice were killed by decapitation at various time intervals of the feeding period. The removed and weighed liver served for enzyme determinations. Carcass weight and liver/body ratio were determined as described in the foregoing communication (1).

Phosphatases

For the quantitative evaluation of phosphatase activities in the mouse liver, the method of Greenstein (6) was used, in which homogenized liver tissue is suspended in distilled water 1 : 10 and extracted for 24 hr. in the cold.

[1] *Manuscript received September 3, 1952, and, as revised, February 4, 1953.*
Contribution from the Division of Animal Pathology, Science Service, Dominion Department of Agriculture (Animal Diseases Research Institute, Hull, Quebec).

Acid and alkaline phosphatase activity was determined in a medium of acetate buffer solution of pH 4.4 and of veronal buffer solution of pH 8.8 respectively. Sodium–glycerophosphate (Eastman) served as substrate. Phosphorus was determined by the method of Fiske and Subbarow (4). Activities are expressed in milligrams of phosphorus liberated per gram dry liver tissue per hour and are related to dry total liver weight and to 100 gm. of carcass weight.

Arginase

Arginase activity was determined after a method employed by Folley and Greenbaum (5). The weighed liver was ground with sand and dispersed in saline, 1 : 10, and 1.5 ml. of the suspension served as enzyme test solution. For the estimation of arginine we used the colorimetric method of Macpherson (7). Arginase values are expressed in 30-min. units related to 1 gm. of dry liver, to the total dry weight of liver, as well as to 100 gm. of carcass weight.

Xanthine Oxidase

Xanthine oxidase activity was determined by the method of Axelrod and Elvehjem (3) adapted to Warburg's manometric technique by Richert, Edwards, and Westerfeld (8); however, the time of dialyzation used by these authors in some of their experiments was reduced from 20 to 3 hr.

The freshly removed and weighed liver was homogenized in a Ten Broeck tissue grinder at a temperature of 3° to 5° C. for approximately five minutes in a ratio 1 : 5 with a refrigerated $M/30$ phosphate buffer solution of pH 7.4 containing an equal number of moles of sodium and potassium. The homogenate was then dialyzed against three liters of the same solution for a period of three hours in the cold. Mouse livers treated in this way did not show the lag period of 40 min. commonly described in rat livers, during which the xanthine has no stimulatory effect on respiration. The stimulated oxygen consumption started almost immediately at the beginning of the experiment, the stopcocks having been closed after an equilibrium period of 10 min. Each Warburg flask contained a final volume of 2 ml. having 0.15 ml. of 40% of potassium hydroxide in the center well sucked into a roll of filter paper. Concurrently, parallel flasks were run for the determination of endogenous respiration without xanthine. Manometer readings were taken every 20 min. for a period of two hours. Xanthine oxidase activity was calculated from the excess of oxygen consumption obtained in the xanthine flask and was expressed in units as indicated by Richert *et al.* (8). Liver protein was determined by precipitating 1 ml. of the tissue homogenate with acetone 1 : 5.

Results

Milligram values of phosphorus liberated by acid phosphatase per gram dry liver tissue per hour were nearly the same in aldrin intoxicated animals as in the controls. The total amount of phosphorus, however, was significantly higher in the former owing to the enlargement of the liver. The same holds true if we relate the milligram values of phosphorus to 100 gm. of carcass weight.

TABLE I

LIVER PHOSPHATASE ACTIVITY

	No. of mice	Days of 50 p.p.m. aldrin feeding	Liver wt., mgm.	L/B*	pH = 4.4		pH = 8.8		pH = 4.4	pH = 8.8
					Mgm. P per 1 gm. dry liver tissue	Mgm. P per total dry liver	Mgm. P per 1 gm. dry liver tissue	Mgm. P per total dry liver	Mgm. P per 100 gm. of carcass	
Control	12	0	1210 ± ·52	0.05 ± .003	2.20 ± .13	0.85 ± .06	0.95 ± .023	0.37 ± .028	4.31 ± 0.30	1.78 ± 0.33
	1	6	2076	.11	2.60	1.50	.43	.25	7.89	1.31
	1	7	1790	.10	2.49	1.24	.69	.34	7.08	1.94
	1	9	1720	.08	2.13	1.25	.69	.41	5.95	1.95
	1	10	2367	.13	1.56	0.94	.39	.27	5.27	1.51
	1	11	2717	.14	2.37	2.00	.76	.34	10.46	1.76
Experiment	1	12	2568	.13	1.87	2.06	.37	.40	10.84	2.11
	1	13	2607	.13	2.83	2.10	.78	.57	11.16	3.03
	1	14	3322	.17	1.67	2.76	.59	.62	13.80	3.10
	1	15	2563	.11	1.73	1.56	.30	.27	6.18	1.17
	1	18	3030	.19	1.93	2.18	.48	.54	13.62	3.37
	1	19	2871	.15	2.08	2.14	.51	.58	11.63	3.15
	1	20	3340	.17	2.16	2.31	.74	.79	11.90	4.07
Total	12				2.10 ± 0.12		0.56 ± .017			

NOTE: $\sigma = \sqrt{(X - \bar{X})^2/(n - 1)}$.

* *Liver/body ratio.*

Alkaline phosphatase activity appeared to be decreased approximately to one half of its normal value in the liver of the intoxicated mouse, calculated from the milligram phosphorus liberated per gram dry liver tissue. Total activity compared with the control values was increased owing to the enlarged liver. The same holds true if we relate the amounts of phosphorus liberated to 100 gm. of carcass weight in the aldrin fed animal.

TABLE II

LIVER ARGINASE ACTIVITY

	No. of mice	Days/p.p.m. aldrin feeding	Liver wt., mgm.	L/B	Unit per 1 gm. dry liver tissue	Unit per dry total liver	Unit per 100 gm. carcass
Control	6	0	1052 ± 37	0.052 ± .0014	1006 ± 56	363 ± 44	1862 ± 35
	1	13/50	2125	.14	1131	714	4760
	1	13/50	2677	.15	1107	990	5562
	1	3/100	2308	.13	924	775	4454
Experiment	1	3/100	1080	.08	816	541	2640
	1	6/100	1906	.11	1010	451	2561
	1	6/100	1574	.10	1175	436	2843
Total	6				1024 ± 57		

Arginase activity, expressed in 30-min. units, was found to be approximately equal in both the experimental and the control animals, again indicating an increase of total activity in the enlarged liver of the intoxicated animal.

Xanthine oxidase activity appeared to be decreased in the liver of mice fed with aldrin. Values calculated for 1 gm. liver protein and for total liver protein both showed a diminution, although the decrease in the latter instance was not so significant owing to the increased liver size of the intoxicated animal.

TABLE III

LIVER XANTHINE OXIDASE ACTIVITY

	No. of mice	Days/p.p.m. aldrin feeding	Liver wt., mgm.	L/B	Unit per 1 gm. liver protein	Unit per total liver protein	Unit per 100 gm. of carcass
Control	6	0	1491 ± 21	0.064 ± .007	329 ± 20	116 ± 36	1405 ± 23
	1	5/75	1380	.072	177	56	931
	1	6/75	1548	.075	151	50	733
Experiment	1	6/75	1984	.098	214	113	1059
	1	8/75	1925	.090	153	74	714
	1	9/75	2655	.150	188	82	1105
	1	12/75	2021	.120	138	92	665
Total	6				170 ± 9		

Discussion

Sulkin and Gardener (9) demonstrated by histochemical investigations that alkaline phosphatase is limited to the nuclear membrane and to the nucleoli in the hepatic cells of the rat. By histochemical and analytical chemical studies of the livers of mice poisoned with diethanolamine, Annau and Manginelli (2) showed that diethanolamine induces an increased production of alkaline phosphatase in the nucleolus of the liver cell, the enzyme eventually filling out the nucleus, whence it diffuses out into the cytoplasm. As a result they suggested that there is a relationship between nucleolar function and alkaline phosphatase activity in the liver. If these observations hold true then changes in alkaline phosphatase activity might be conceived as an alteration in the nucleoli. Consequently the reported decrease in alkaline phosphatase activity in the liver of aldrin-intoxicated mice may also be interpreted in this sense.

The interpretation of the decrease in xanthine oxidase activity presents some difficulty, as this enzyme appears to be influenced by a great variety of substances and dietary factors. The decrease may be induced by a specific toxic effect of aldrin, or it may also be due to a general depletion of flavoprotein enzymes.

Acid phosphatase and arginase activity however seemed to be unaltered by aldrin, as their relative concentration, in comparison to the controls, remained unchanged, whereas their absolute amount increased, corresponding to the liver enlargement.

Summary

Four liver enzymes, selected at random for investigation in connection with prolonged feeding of mice with sublethal doses of aldrin, gave the following results. Acid phosphatase and arginase activities, in comparison with control animals, appeared unaltered. Alkaline phosphatase and xanthine oxidase activities were decreased in their relative amounts, and the latter also in its absolute amount.

Acknowledgments

Thanks are due to Miss Grace Walker for technical assistance with the chemical aspects of this work and to Dr. Chas. A. Mitchell, Chief of the Division, for his interest in connection with the problem.

References

1. Annau, E., Konst, H., and Plummer, P. J. G. Can. J. Med. Sci. 30 : 463. 1952.
2. Annau, E. and Manginelli, A. Nature, 166 : 815. 1950.
3. Axelrod, A. E. and Elvehjem, C. A. J. Biol. Chem. 140 : 725. 1941.
4. Fiske, C. H. and Subbarow, T. J. Biol. Chem. 66 : 375. 1925.
5. Folley, S. J. and Greenbaum, A. L. Biochem. J. 46 : 261. 1947.
6. Greenstein, J. P. J. Natl. Cancer Inst. 2 : 511. 1942.
7. Macpherson, M. T. Biochem. J. 36 : 59. 1942.
8. Richert, D. A., Edwards, S., and Westerfeld, W. W. J. Biol. Chem. 181 : 255. 1949.
9. Sulkin, N. H. and Gardener, I. H. Anat. Record, 100 : 143. 1948.

THE METABOLISM OF C^{14}-UREA IN THE RAT[1]

By S. H. Zbarsky and W. D. Wright[2]

Abstract

Urea, labelled with carbon^{14}, was synthesized and administered to rats by intraperitoneal injection. The excretion of the carbon^{14} was followed by analysis of the urine and expired carbon dioxide for radioactivity at various times after the injection and the distribution of the isotope was determined by analysis of organs, blood, and carcass. A portion of the injected urea was rapidly metabolized, up to 17% of the isotope being excreted in the expired air after three hours and 30% after 48 hr. The highest output of $C^{14}O_2$ occurred during the second hour after injection. Most of the remaining isotope was excreted in the urine as urea. After three hours only a small percentage of the injected carbon^{14} was present in the kidney, liver, and blood, largely as urea. There appeared to be little incorporation of the isotope into the tissues.

Introduction

For many years it had been assumed that urea was merely a metabolic product of protein metabolism and, once formed, underwent no further metabolism, being excreted in the urine. The work of Bloch (4) appeared to confirm this view. Bloch fed rats urea labelled with N^{15} and from a study of the excretion of the isotope concluded that urea lacked any metabolic activity and did not participate in any intermediary reaction. More recently Leifer, Roth, and Hempelmann (7) studied the metabolism of C^{14}-labelled urea in mice and found that within 48 hr. after injection of the material, 20.8% of the radioactivity appeared as carbon dioxide in the expired air.

As part of a program involving the synthesis of various radioactive purines and pyrimidines C^{14}-urea was prepared and it was felt of interest to study the metabolism of this material in the rat. It was found that after a single injection of C^{14}-urea into the rat, a considerable fraction of the radioactivity is rapidly excreted as expired carbon dioxide, the output reaching a maximum during the second hour. Most of the remainder of the C^{14} is excreted in the urine where it is present only as urea. Very little was found fixed in the tissues.

Experimental

Preparation and Counting of Radioactive Samples

Unless otherwise stated, all radioactive material was converted to barium carbonate for counting. Organic matter was oxidized to carbon dioxide using the method of Armstrong and Schubert (2) except that the carbon dioxide

[1] *Manuscript received January 28, 1953.*

Contribution from the Department of Biochemistry, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia. A preliminary report of this investigation was presented at the Western Regional Meetings, Medical Research Division, National Research Council, at Vancouver, on February 7, 1952.

[2] *Taken from a thesis presented by W. D. Wright to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Arts.*

formed was trapped in carbonate-free sodium hydroxide solution (5) instead of a saturated solution of barium hydroxide. The carbonate formed was then precipitated as barium carbonate (9), centrifuged, washed, and filtered on a brass counting dish prepared as described by Armstrong and Schubert (1). The sample was dried and counted under an end mica window Geiger-Müller tube (window thickness, 1.6 mgm. per cm.^{2}) connected to a scaling unit (Model 163, Nuclear Instrument and Chemical Corporation). Sufficient counts were taken with most of the samples to give a counting error not greater than 5% and, with more highly active samples, 1%.

When feasible, enough material was oxidized to give an "infinitely thick" sample of barium carbonate (5) for counting. If insufficient material was available, nonisotopic carbonate was added before precipitation or, in some cases, carrier material was added before oxidation to ensure the formation of a thick sample. The usual corrections were applied to all counts (5).

C^{14}-Urea

The C^{14}-urea used was prepared from radioactive barium carbonate according to the method of Zbarsky and Fischer (10). The following experiments showed that no other radioactive material was present in detectable amounts.

The radioactive material was subjected to filter paper partition chromatography (7) using, as developing solutions, butanol saturated with water in one experiment and, in another, a solution of butanol, ethanol, and water in equal volumes. The chromatograms were allowed to develop overnight and the paper strips were removed from the chromatography jars and dried in air. The strips were then placed in contact with Blue Brand No-Screen X-ray film and kept in a dark room for seven days. On developing the film, only one spot appeared in each case, indicating only one radioactive component. The R_f values for these spots were 0.53 when the butanol–water solvent was used and 0.26 when the butanol–ethanol–water solvent was used.

The chromatographic behavior of the radioactive material was also compared with that of authentic urea using strips of filter paper on which samples of each had been spotted. Three different solvents were used; butanol saturated with water, butanol–ethanol–water (1 : 1 : 1 by volume), and tertiary butanol – concentrated hydrochloric acid – water (1 : 1 : 1 by volume). In each case, after the chromatograms were allowed to develop overnight, the filter paper strips were removed from the chromatography jar and dried in air. In order to locate the urea on the paper a modification of the procedure of Berry (3) was used. The paper strips were sprayed with a solution of commercial bleach (approximately 5% sodium hypochlorite solution) and while still wet were sprayed with a 5% solution of phenol in ethanol. Immediately, yellow spots appeared on the paper indicating the position of the urea. On drying, the spots assumed a mottled yellow-green color and little fading of the spots was observed over a period of one month. The R_f values obtained in these experiments are shown in Table I.

TABLE I

R_f VALUES OBTAINED BY PAPER CHROMATOGRAPHY OF RADIOACTIVE AND AUTHENTIC UREA USING VARIOUS SOLVENTS

Solvent	Butanol saturated with water	Butanol–ethanol–water 1 : 1 : 1 by vol.	Butanol-HCl-water 1 : 1 : 1 by vol.
Authentic urea	0.24	0.55	0.67
Radioactive urea	0.24	0.54	0.67

The results shown in Table I and the close agreement between the values shown in Columns 2 and 3 of the table and the R_f values obtained in the previous experiment indicate that the radioactive material consisted only of urea.

Further proof was obtained by comparing the radioactivity liberated from the material by oxidation to carbon dioxide with that obtained on treatment with urease. Hydrolysis by urease was carried out by treating a solution of the urea in phosphate buffer of pH 7.4 with glycerol–urease solution (6). The mixture was then acidified strongly with phosphoric acid and the evolved carbon dioxide swept into a carbonate-free solution of sodium hydroxide (5). The carbonate was precipitated and counted as barium carbonate. To each of six aliquots of an aqueous solution of the C^{14}-urea were added 50 mgm. of nonisotopic urea in order to obtain a thick sample for counting. Three of the aliquots were then evaporated to dryness *in vacuo* over concentrated sulphuric acid and the residues oxidized to carbon dioxide. The remaining three samples were treated with urease. The results obtained are shown in Table II.

TABLE II

RECOVERY OF RADIOACTIVITY FROM C^{14}-UREA AFTER OXIDATION TO CARBON DIOXIDE AND AFTER HYDROLYSIS WITH UREASE

Treatment of samples	Weight of $BaCO_3$ obtained, mgm.	Radioactivity, c.p.m.
Evaporation to dryness and oxidation of residue to CO_2	165.2	6252
	164.5	6240
	164.5	6300
		Average 6264
Hydrolysis with urease	165.5	6250
	167.6	6295
	165.0	6240
		Average 6262

Metabolism Experiments

(*i*) *General Procedure*

The radioactive urea was dissolved in 0.9% sodium chloride solution and the solution was administered to the rat by intraperitoneal injection. The animal was then placed in a closed metabolism apparatus which permitted the collection of urine and faeces separately and the expired air was swept out continuously with a stream of carbon dioxide-free air into traps containing carbonate-free sodium hydroxide solution. It was possible to collect the expired carbon dioxide and urine for any desired interval without interrupting the flow of air through the apparatus.

At the end of each experiment, the animal was removed, anaesthetized, and bled as completely as possible from the carotid artery. Kidneys, liver, spleen, stomach, and intestines were removed and these organs and the faeces were dried to constant weight at 100° C. When necessary the dried material was extracted with an alcohol–ether solution in a Soxhlet apparatus to remove fatty material and dried again. The dried residues were pulverized and the tissue powder counted directly. If a significant amount of radioactivity was detected, weighed samples of the powdered residue were oxidized to carbon dioxide, which was counted as barium carbonate.

The remainder of the carcass was dissolved in hot, concentrated, potassium hydroxide solution. The solution was cooled, made up to one liter, and suitable aliquots were evaporated to dryness and the residues oxidized to carbon dioxide, which was counted as barium carbonate.

The urine was collected at various times after the injection of the C^{14}-urea. Each urine collection was made up to a suitable volume in a volumetric flask and the radioactivity determined in aliquot samples by oxidation of the residues after evaporation to dryness and also by hydrolysis with urease. In no case was any difference observed in the radioactivity liberated by these two methods. This finding showed that all of the C^{14} excreted in the urine was present as urea.

As stated above, the air breathed out by the animal was swept out by a stream of carbon dioxide-free air through towers containing carbonate-free sodium hydroxide solution to trap out the expired carbon dioxide. Two towers were so arranged that it was possible to collect the expired carbon dioxide in one tower for any given period and then to switch the stream of air from the apparatus into the other tower. The sodium hydroxide was then drained from the first tower into a volumetric flask and the tower was washed thoroughly with boiled distilled water, the washings being drained into the volumetric flask. The sodium hydroxide solution was made up to volume and the carbonate precipitated from aliquots as barium carbonate which was counted.

(*ii*) *Experiment 1*

In this experiment, which was of a preliminary nature, urea of low specific activity was used. A female albino rat, weighing 190 gm., was given an intraperitoneal injection of 0.5 ml. of a saline solution of C^{14}-urea containing

500 mgm. of urea per ml. Since the specific activity of the urea was 20 counts per minute per mgm., a total of 5000 c.p.m. of C^{14} was administered. Expired air was collected for hourly intervals and urine collected once at the end of the experiment. Four hours after it had received the injection, the rat was removed from the metabolism apparatus, anaesthetized, and its blood and organs removed and treated as described. The results obtained after assay of the various samples for radioactivity are shown in Table III.

TABLE III

CARBON^{14} CONTENT OF THE TISSUES AND EXCRETA OF A RAT KILLED FOUR HOURS AFTER INTRAPERITONEAL INJECTION OF 250 MGM. OF C^{14}-UREA WITH A SPECIFIC ACTIVITY OF 20 C.P.M./MGM. UREA. TOTAL COUNTS INJECTED, 5000 PER MIN.

Material examined	Radioactivity found	
	Total c.p.m.	Per cent of injected counts
Expired air, 1st hour	220	4.4
Expired air, 2nd hour	320	6.4
Expired air, 3rd hour	300	6.0
Expired air, 4th hour	210	4.2
Liver, kidney, spleen	950	19.0
Blood	Trace	—
Stomach, intestine, and faeces	Trace	—
Carcass	—	—
Urine	2900	58.0
Total	4900	98.0

As shown in the above table, 21% of the C^{14} injected as urea was excreted in the expired air. This represents 37.6% of the total C^{14} excreted during the course of the experiment, the remainder being excreted in the urine. The C^{14} in the urine was present only as urea. The output of C^{14} in the expired air was highest during the second hour after the injection of the radioactive urea.

(*iii*) *Experiment 2*

In this experiment, C^{14}-urea of much higher specific activity was used, making it possible to inject an amount of urea more closely approaching the physiological range. A male albino rat, weighing 250 gm., was injected intraperitoneally with 0.5 ml. of a saline solution of urea containing 20 mgm. of urea per ml. The urea had a specific activity of 26,900 c.p.m. per mgm. The animal was placed in the metabolism apparatus and supplied with food and water ad libitum. Expired carbon dioxide was collected at 1, 2, 3, 6, 12, 24, 30, 36, and 48 hr. and urine at 6, 12, 24, 30, 36, and 48 hr. after injection of the urea. The animal was removed at the end of the 48th hour and anaesthetized. Before the animal was bled, a small amount of heparin was injected into the jugular vein in order to prevent the collected blood from clotting. The various organs, expired carbon dioxide, urine, carcass, and

faeces were analyzed for radioactivity as already described. In this experiment, the stomach and intestines of the animal contained considerable amounts of fat. These tissues were therefore extracted in a Soxhlet apparatus with an ethanol–ether solution (3 : 1 by volume) and dried again before they were counted. No radioactivity was detected in the fatty material removed during the extraction.

The blood was centrifuged and the plasma and the red cells were dried separately and counted.

In Table IV are shown the recoveries of radioactivity from the tissues examined.

TABLE IV

CARBON^{14} CONTENT OF THE TISSUES AND EXCRETA OF A RAT KILLED 48 HR. AFTER INTRAPERITONEAL INJECTION OF 10 MGM. OF C^{14}-UREA WITH A SPECIFIC ACTIVITY OF 26,900 C.P.M./MGM. UREA. TOTAL COUNTS INJECTED, 269,000 per min.

Material examined	Radioactivity found	
	Total c.p.m.	Per cent of injected counts
Expired air	81,300	30.2
Urine	170,180	63.2
Liver, kidneys, spleen, stomach, intestines	800	0.3
Blood	Trace	—
Faeces	1000	0.4
Carcass	13,070	4.8
Total	266,350	98.9

During the 48-hr. period of the experiment, 30% of the injected C^{14} appeared in the expired carbon dioxide, indicating an extensive metabolic degradation of the urea. Sixty-three per cent of the C^{14} was excreted in the urine as urea, and almost 5% was still present in the carcass material. The results also indicate that 48 hr. after a single injection of C^{14}-urea there is little radioactivity present in the blood.

A more detailed presentation of the excretion of C^{14} during the course of this experiment is given in Table V, which shows that the excretion of radioactive carbon dioxide was rapid, beginning shortly after the injection of the radioactive urea. The output of radioactive carbon dioxide, as in the previous experiment, was highest during the second hour after the injection, after which it fell, relatively little appearing in the expired air after 12 hr. The excretion of C^{14} in the urine was very high during the first six hours of the experiment, 53.6% of the total urinary excretion of C^{14} appearing during that period. After the 12th hour the excretion of C^{14} in the urine fell sharply. The large amount of C^{14} and the high specific activity of the urinary urea excreted during the first 12 hr. after the injection indicated that a considerable portion of the injected urea was excreted directly after absorption from the site of injection.

TABLE V

EXCRETION OF C^{14} IN EXPIRED AIR AND URINE BY A RAT AFTER INTRAPERITONEAL INJECTION OF 10 MGM. OF C^{14}-UREA. (269,000 C.P.M.)

Period of collection, hr.	Radioactivity excreted			
	Urine		Expired air	
	Total c.p.m.	Specific activity, c.p.m./mgm. C	Total c.p.m.	Specific activity, c.p.m./mgm. C
0- 1	—	—	10,200	90.0
1- 2	—	—	16,700	124.0
2- 3	—	—	13,700	78.0
3- 6	—	—	19,600	40.0
0- 6 (urine only)	92,340	5660	—	—
6-12	69,560	1480	12,800	9.0
12-24	4420	98	4000	1.4
24-30	1360	40	1500	1.0
30-36	1770	37	1100	0.7
36-48	730	19	1700	0.6
Total	170,180	—	81,300	—

Fig. 1 shows the cumulative excretion of C^{14} in the urine and expired air during the experiment. From the curve it can be seen that the time required for the excretion of 50% of the injected C^{14} was approximately five hours, which agreed with the results obtained with mice by Leifer, Roth, and Hempelmann (7).

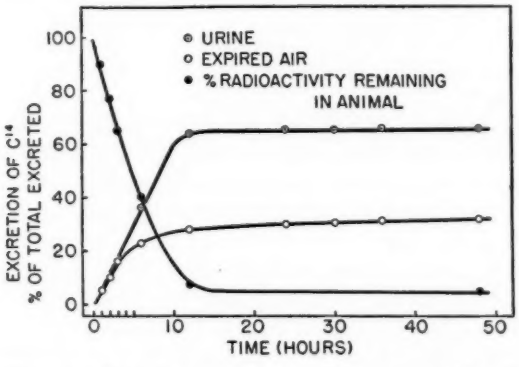


FIG. 1. Excretion of C^{14} in urine and expired air by a rat which received a single intraperitoneal injection of 10 mgm. of C^{14}-urea with a specific activity of 26,900 c.p.m. per mgm.

(iv) Experiment 3

The purpose of this experiment was to study the excretion and the distribution of radioactivity in various tissues of the rat shortly after the animal had received an injection of C^{14}-urea, when the metabolic breakdown of the urea to carbon dioxide was proceeding at a relatively high rate.

One-half milliliter of a saline solution of urea containing 20 mgm. of urea per ml. was injected intraperitoneally into a male albino rat, weighing 250 gm. The urea used in this experiment had a specific activity of 31,250 c.p.m. per mgm. so that a total of 312,500 counts was injected. The rat was placed in the metabolism apparatus and expired carbon dioxide was collected hourly for three hours and urine at the end of three hours. No faeces were excreted during this time. At the end of the three-hour period the rat was anaesthetized and heparin injected into the jugular vein. The blood was then collected and the organs excised. The expired carbon dioxide, urine, spleen, stomach, intestines, and carcass were assayed for radioactivity in the manner already described.

Liver and kidney were minced separately in a Waring blendor with an ice-cold 5% solution of trichloroacetic acid and the mixture centrifuged. The residue was washed with small portions of the cold trichloroacetic acid solution and the washings were combined with the original supernatant solution. The trichloroacetic acid extract was in turn extracted with ether and the solution remaining after this extraction was assayed for radioactive urea by the urease method. The ether extract was also tested for radioactivity by removing the ether and counting the dried residue directly. No radioactivity was found in the ether extract and only traces were in the protein residue remaining after extraction of the tissue with the trichloroacetic acid.

Radioactive urea in the whole blood was determined by the urease technique carried out on a protein-free filtrate of the blood. In order to determine whether radioactive carbon dioxide was present in the blood, 1-ml. aliquots of the whole blood were pipetted into large test tubes connected to similar tubes containing carbonate-free sodium hydroxide solution. The blood was

TABLE VI

CARBON^{14} CONTENT OF TISSUES AND EXCRETA OF A RAT KILLED THREE HOURS AFTER INTRAPERITONEAL INJECTION OF 10 MGM. OF C^{14}-UREA WITH A SPECIFIC ACTIVITY OF 31,260 C.P.M./MGM. UREA. TOTAL COUNTS INJECTED, 312,600 per min.

Material examined	Radioactivity found	
	Total c.p.m.	Per cent of injected counts
Expired air, 1st hour	11,060	3.5
Expired air, 2nd hour	11,250	3.6
Expired air, 3rd hour	7400	2.3
Urine	99,000	31.7
Blood urea	4300	1.3
Blood CO_2	110	0.03
Blood protein	—	—
Kidney urea	4300	1.3
Liver urea	3300	1.0
Kidney and liver protein	Trace	—
Carcass	140,000	45.1
Total	280,720	89.8

acidified with hydrochloric acid and aerated to carry evolved carbon dioxide over into the sodium hydroxide solution. The carbonate was then precipitated, after the addition of nonisotopic sodium carbonate, and counted as barium carbonate. The protein of the blood was precipitated and counted directly.

The results of this experiment are shown in Table VI. As indicated in the table, only traces of radioactivity were found in the proteins of the tissues examined or in the fat extracted from the stomach and intestines. Both liver and kidney contained considerable amounts of radioactive urea, the kidney content being slightly higher than that of the liver. No experiment was carried out to determine the nature of the radioactive material found in the carcass but it was thought to be largely unabsorbed urea.

Ten per cent of the injected radioactivity was not accounted for. However, this loss may be explained by the observation that a small amount of urine, containing urea of high specific activity, was lost after the rat had been removed from the metabolism cage. Furthermore, some radioactivity was lost in the expired air during the period when the animal was being anaesthetized and bled.

Discussion

From the experiments that have been described, it is evident that in the rat considerable metabolism of urea to carbon dioxide occurs. In Experiment 2, for example, it was found that over a period of 48 hr. following a single injection of C^{14}-urea to a rat 30% of the injected C^{14} appeared as carbon dioxide in the expired air. It is possible that not all of this radioactive carbon dioxide arose directly from the injected urea but that some may have arisen indirectly from the metabolic degradation of compounds which had incorporated C^{14} from the injected urea. In all three experiments a rapid excretion of radioactive carbon dioxide was observed, 17% of the injected C^{14} being expired during the first three hours in Experiment 1, 15% in Experiment 2, and 9% in Experiment 3. The number of counts excreted and the specific activity of the expired carbon dioxide reached a maximum in each experiment during the second hour after the injection of the labelled urea. This finding is illustrated in Fig. 2.

Most of the administered C^{14} was excreted in the urine. Examination of this C^{14} by the methods described showed that the radioactivity was present in the urine only as urea. The excretion of radioactivity in the urine was very rapid, amounting to as much as 31.7% of the injected C^{14} in three hours (Experiment 3). From the very high specific activity of the urea excreted during the first few hours after injection of the C^{14}-urea it was concluded that a considerable fraction of the injected material had been carried directly to the kidneys and excreted without entering into any metabolic reaction. Radioactive urea continued to be excreted, however, for at least 48 hr. after injection as shown in Experiment 2. Whether this urea consisted of injected urea which had escaped degradation to carbon dioxide or arose indirectly from degradation of the injected urea and reincorporation of the C^{14} into urea

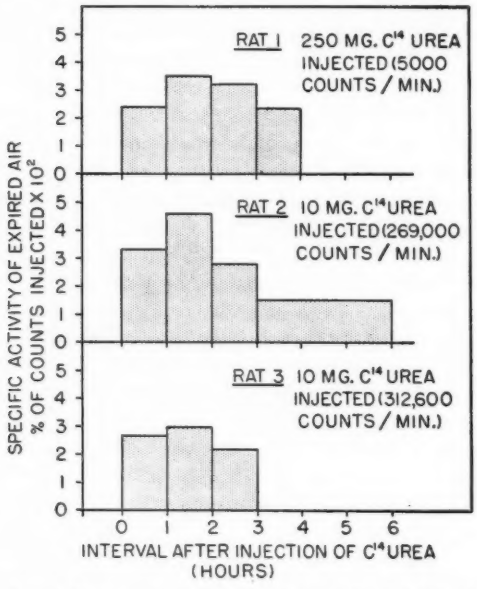


FIG. 2. Excretion of C^{14} in expired CO_2 after intraperitoneal injection of C^{14}-urea.

through further metabolic reactions is not known. Of interest are the results shown in Table V indicating that the specific activity of the excreted urea during the 48-hr. period following injection of C^{14}-urea was at all times higher than that of the expired carbon dioxide, since from the experiments of McKenzie and du Vigneaud (8) it might be expected that the specific activities would approach a common value.

In the experiments described there appeared to be little incorporation of the injected C^{14} into the tissues. Three hours after injection the radioactivity present in liver, kidney, and blood was present almost entirely as urea. A large percentage of the injected radioactivity was present in the carcass material, presumably as unabsorbed urea. Forty-eight hours after injection, barely 5% of the injected radioactivity could be detected in the carcass.

Acknowledgment

It is a pleasure for the authors to express their thanks to the National Research Council for financial assistance through a grant-in-aid.

Summary

1. A study has been made of the excretion of C^{14} and its distribution in the tissues of rats receiving a single intraperitoneal injection of C^{14}-urea.

2. Following the administration of the urea there was a rapid appearance of radioactive carbon dioxide in the expired air of the animals. The radioactivity of the carbon dioxide reached a maximum during the second hour after injection. During the first three hours after injection of C^{14}-urea as much as 17% of the radioactive carbon was excreted as carbon dioxide and, in a 48-hr. experiment, 30% of the injected radioactivity was excreted as carbon dioxide.

3. Most of the injected C^{14} was excreted in the urine. There was a very rapid excretion of urea of high specific activity which indicated a direct excretion of some injected C^{14}-urea during the first few hours of the experiment. Radioactive urea continued to be excreted for as long as 48 hr. after injection.

4. There was little evidence of incorporation of the C^{14} of the injected urea into the tissues. It was found that three hours after injection of C^{14}-urea the radioactivity in liver, kidney, and blood was present almost entirely as urea. The kidneys were highest in radioactive content and blood was lowest. Forty-eight hours after injection of the radioactive urea, approximately 5% of the C^{14} remained in the carcass material.

References

1. ARMSTRONG, W. D. and SCHUBERT, J. Anal. Chem. 20 : 270. 1948.
2. ARMSTRONG, W. D. and SCHUBERT, J. Anal. Chem. 20 : 1120. 1948.
3. BERRY, H. K. Univ. Texas Pub. No. 5109 : 88. 1951.
4. BLOCH, K. J. Biol. Chem. 165 : 469. 1946.
5. CALVIN, M., HEIDELBERGER, C., REID, J. C., TOLBERT, B. M., and YANKWICH, P. F. Isotopic carbon. John Wiley & Sons, Inc., New York. 1949.
6. KOCH, F. C. and HANKE, M. F. Practical methods in biochemistry. 5th ed. The Williams & Wilkins Company, Baltimore. 1948.
7. LEIFER, E., ROTH, L. J., and HEMPELMANN, L. H. Science, 108 : 748. 1948.
8. MCKENZIE, C. G. and DU VIGNEAUD, V. J. Biol. Chem. 172 : 353. 1948.
9. REGIER, R. B. Anal. Chem. 21 : 1020. 1949.
10. ZBARSKY, S. H. and FISCHER, I. Can. J. Research, B, 27 : 81. 1948.

PARALLEL STUDIES OF COMPLEMENT AND BLOOD COAGULATION

VIII. THE EFFECT OF CHOLINE AND METHIONINE ON THE CHANGES INDUCED IN GUINEA PIGS BY THE INJECTION OF CARBON TETRACHLORIDE[1]

By Christine E. Rice[2], Paul Boulanger[2], and P. J. G. Plummer[2]

Abstract

A marked prolongation of the two-stage clotting time of the plasma was produced in guinea pigs by the repeated injection of small weekly doses of carbon tetrachloride. Ac-globulin (labile factor), as well as fibrinogen and prothrombin, was reduced. A significant fall in whole complement titer also occurred; of the four major complement components, C′ 2 and C′ 3 were the most affected. Subsequent and concurrent daily feedings of choline chloride or methionine reduced the clotting time of the plasma of certain of the carbon tetrachloride treated animals but not to normal levels; they had no significant influence on complement titer. Previous and concurrent daily feedings of choline chloride ameliorated the fatty liver condition and gave some protection against the decrease in the coagulability of the blood induced by the drug; the decline in complement titer was not prevented. Ethionine augmented the changes induced by carbon tetrachloride.

Introduction

As normal protein constituents of the blood, certain of the major components of the coagulation and complement systems are affected by factors that influence the general synthesis of proteins in the animal body. In an attempt to obtain a better understanding of the nature and function of both of these important systems and of ways of maintaining their activities at optimal levels, we have been devoting considerable attention to a study of the changes in both properties that accompany the liver damage in guinea pigs resulting from the administration of substances such as carbon tetrachloride (21), chloroform (22), gum acacia (23), and ethionine (24), or from a high cholesterol diet (7). However, since pathological changes were frequently observed in other organs as well as the liver, it did not follow that the observed effects could be attributed entirely to the liver damage.

Of the agents employed, carbon tetrachloride and ethionine induced the most marked and consistent decline in coagulability and complement titer. The four major complement components responded somewhat differently to the two drugs. Analysis of the serum protein content by salt fractionation (18), also indicated differences in the response (21, 24). Whereas the repeated injection of small amounts of carbon tetrachloride was usually followed by a fall in the total protein and albumin content of the serum, little change was usually recorded in the values of either in the ethionine treated animals. Furthermore, serum "euglobulin" tended to increase in the former

[1] *Manuscript received February 18, 1953.*

Contribution from the Division of Animal Pathology, Canadian Department of Agriculture, Animal Diseases Research Institute, Hull, Quebec.

[2] *Division of Animal Pathology, Animal Diseases Research Institute.*

group, to decrease in the latter. This suggested that although both substances had caused a fatty infiltration of the liver, their effect on protein physiology had not been the same. It seemed accordingly that further comparative studies of animals injected with the two agents might provide information of a fundamental nature in regard to the natural synthesis of the components of both systems.

The first step has been to determine whether lipotropic substances such as choline or methionine, by preventing or ameliorating the fatty liver condition induced by carbon tetrachloride or ethionine, will at the same time protect against these changes in blood coagulability and complement titer. The present paper will deal with the observed effects in the carbon tetrachloride treated guinea pigs; the next paper in this series will present the results of a similar study in animals to which ethionine has been administered. Some preliminary studies of the effects of three vitamins, E, K, and B_{12}, have been made and will be summarized briefly as suggestions for further investigations.

Literature Review

The amount of fat present in the liver depends upon a balance between factors tending to increase liver fat and those tending to decrease it. In the first category are included the lipid synthesis by the liver cells themselves, a mobilization from fat depots elsewhere, and absorption from the intestine. A decrease in liver fat results from lipid catabolism in this organ and a transport of fat from other areas in the body. The fatty liver found in carbon tetrachloride poisoning has been attributed to a more rapid mobilization of fat from the body stores than can be dealt with immediately by the liver cells (3, 27). In the ethionine-injected animals which do not show the rapid emaciation usually observed in those receiving carbon tetrachloride, the accumulation of fat in the liver may possibly be the result of a lack of transport.

The histological appearance of the liver of rats following the injection of the two drugs differs in regard to the distribution of the fat. After carbon tetrachloride, there tends to be a central zone of necrosis, with a succeeding zone occupied by large and small fat droplets (12). Ethionine, by contrast, produces a fatty liver without necrosis; the fat droplets are smaller, and, although widely distributed throughout the liver, usually predominate in the peripheral zone (19). The distribution of fat in the livers of guinea pigs and rabbits injected with ethionine appears very similar to that described for the rat. It has been suggested that the liver changes induced by ethionine resemble those found in certain dietary conditions rather than those elicited by hepatotoxins (19).

The very extensive literature on the role of various lipotropic substances in controlling the accumulation of fat in the liver has been discussed in a number of recent reviews and publications (1, 5, 16, 20, 28). Of the natural lipotropic substances, choline and methionine are the two of the most importance. The lipotropic effect of choline has been attributed in part to a stimulation of

phospholipid formation (5, 10, 14, 26). Methionine contributes methyl groups for the formation of choline but has other important physiological functions in addition to lipotropism, as in the *in vivo* transfer of methionine-S to cysteine-S (1).

The response of fatty livers to lipotropic agents is dependent on the character of the lipids they contain (5). Both choline and methionine fail to avert fatty liver in starved rats, but are effective in the treatment of fatty liver resulting from a high fat – low protein diet (5). Very large amounts are required to protect against, or ameliorate, that produced by toxic substances such as chloroform or carbon tetrachloride (2). Methionine, but not choline, has been shown to prevent the appearance of fatty livers in ethionine-injected rats, which would suggest that the protective effect of methionine in this instance may depend primarily upon other functions than methylation (13). Because of the reported lack of choline oxidase in the livers of guinea pigs (4, 15), it does not necessarily follow that choline and methionine will have effects on these animals similar to those observed in other species with large amounts of this enzyme. However, *in vivo* experiments with radioactive tracers have suggested that choline methyl is labile in the guinea pig, although quantitatively less active than betaine methyl (9).

Methods

Treatment of Guinea Pigs

Experiment 1

Beginning April 28, 1951, 35 male guinea pigs, weighing 500 to 700 gm., were given five weekly, intraperitoneal injections of 0.2 ml. carbon tetrachloride. They were bled two days after the last injection along with six untreated control animals. All of the injected animals had lost weight, mean decline 94 gm., whereas the controls showed a mean gain of 25 gm. during the same period. The 30 surviving treated animals were divided according to weight into six groups of five animals each.

Beginning May 8 and continuing to June 5, the second group received 100 mgm. choline chloride daily by mouth, the third group 100 mgm. *dl*-methionine. The fourth group was fed 1 mgm. vitamin K daily, the fifth 7.5 mgm. vitamin E, the sixth group received 3.5 μgm. vitamin B_{12} intramuscularly.* During the time these supplements were being given the animals received two further injections of 0.2 ml. carbon tetrachloride, on May 31 and June 5 respectively. Second bleedings were taken June 6.

The majority of guinea pigs continued to lose weight irrespective of whether dietary supplements were being given or not; some died before the second bleeding date. After the second bleeding no further injections of carbon tetrachloride or dietary supplements were given. Third bleedings were collected June 18 or 19, at which time all surviving animals showed a definite gain in weight.

* *The vitamin preparations used were: Natural vitamin K (K-SAN) from the Synthetic Drug Company Ltd., Toronto; vitamin B_{12} (Rubramin) from E. R. S. Squibb and Sons; and vitamin E (REX) as stabilized wheat germ oil from Vio Bin Ltd., St. Thomas, Ontario.*

Experiment 2

On July 30, 50 male guinea pigs, weighing 650 to 870 gm., were divided on the basis of weight into 5 groups of 10 each. The third group of animals was fed 100 mgm. choline chloride from this date to July 13 when they were bled from the heart; the daily feedings were continued until July 19 and a second bleeding taken July 20. Five normal animals from the first group were bled on the same dates. The fourth and fifth groups were fed 100 mgm. *dl*-methionine or ethionine respectively from July 6 to July 23 and bled July 16 and 24 along with the remaining five animals from the first group.

Four days after feeding was begun, the third, fourth, and fifth groups, and the hitherto-untreated second group were injected subcutaneously with 0.2 ml. carbon tetrachloride; two further doses were given two and four days later. The second group was bled on the same dates as the third group.

Five additional guinea pigs received 16 daily feedings of 100 mgm. ethionine but no carbon tetrachloride as a control of the effect of ethionine alone.

Coagulation Tests

Two-stage prothrombin determinations were made on plasma diluted 1 : 25 as described in our previous papers (21-24). The test is carried out in three steps: Stage I, stage II, and stage II (modified). For stage I, the mixture consists of 0.5 ml. of a 1 : 25 saline dilution of plasma, 0.5 ml. of barbitone buffer of pH 7.3, and 1.0 ml. of calcium chloride – thromboplastin reagent. The mixture is prepared in tubes previously placed in an illuminated glass water bath at 37° C. A stop watch is started immediately after the thromboplastin has been added. After 45 sec., 0.2 ml. of the mixture is transferred to another tube for the stage-II test. The time required for clotting in the residual stage-I mixture is recorded.

In the stage-II test, 0.1 ml. of ice-cold fibrinogen (sheep) is added to the 0.2 ml. of the mixture from stage I. The clotting time is noted. In stage II (modified), the 1 : 25 dilution of plasma is prepared in a 1 : 25 to 1 : 100 dilution of prothrombin-free guinea pig serum treated according to the method of Ware and Seegers (29). The mixture is made as described for stage I and the 0.2 ml. sample removed after the usual 45 sec. conversion time. Fibrinogen is added as in stage II (unmodified) and the clotting time determined. The tubes are rotated gently at intervals in all tests.

Complement-fixation Tests

Complement titrations were made on serum within two to three hours of bleeding: the individual components were titrated using reagents prepared from guinea-pig complement (6, 25). The amount of complement required for 50% hemolysis was estimated in each test and the titer expressed in units per ml. of serum. Because of the variation that may be observed in complement titers at different seasons of the year, the dates of bleeding have been given for each experiment. In view of this seasonal tendency, and because of the fact that new sets of test reagents prepared for each experiment may give

somewhat different results, all titer values should be considered in relation to those obtained for the untreated controls bled on the same date before they are compared with those recorded for previous or subsequent bleedings from the same group of animals.

Results

All of the animals that died during the preliminary treatment with carbon tetrachloride in the first experiment exhibited extensive fatty degeneration of the liver. The dosage of carbon tetrachloride was kept low to avoid necrosis if possible, since the liberation of toxic split products from the tissue proteins would introduce an additional complicating factor. A number of guinea pigs in each group were sacrificed after the third bleeding. The livers of three untreated controls and two of the choline treated animals appeared normal; those of three carbon tetrachloride treated controls, without dietary supplement, and three of the vitamin K group were almost normal with only a few scattered cells containing small vacuoles.

All of the carbon tetrachloride treated guinea pigs in the second experiment also showed a decline in weight irrespective of whether they had been fed choline or methionine previously and concurrently. The livers of the animals that were sacrificed after the first bleeding appeared fatty and on microscopic examination gave evidence of a fatty degeneration of varying extent. Choline seemed to have a somewhat greater protective effect than methionine. In the livers of two choline-fed animals killed July 13, as much as two thirds of the tissue appeared normal, whereas the livers of four methionine-fed guinea pigs sacrificed on the same date showed a slight to severe fatty degeneration of the peripheral and medial zones of all lobules. Two with ethionine supplement displayed a severe and very diffuse fatty degeneration with very few normal cells. Marked fatty changes were also recorded in the livers of the ethionine controls without carbon tetrachloride treatment. The livers of all ethionine treated animals sacrificed after the third bleeding still showed considerable fatty change.

Effect on Coagulative Activity

Experiment 1

The five preliminary weekly injections of carbon tetrachloride induced marked changes in the coagulative properties of the diluted plasma of all guinea pigs. The mean stage-I clotting time of the first bleeding from the 30 surviving animals was 413 sec. (range 267-725 sec.; $\sigma = 99$) as compared with a mean value of 158 sec. (range 136-197 sec.; $\sigma = 79$) for the six untreated controls. The mean stage-II clotting time for the normals was 37 sec. (range 25-47 sec.; $\sigma = 9.3$ sec.) and for the treated animals 299 sec. (range 148-635 sec.; $\sigma = 141$ sec.). The addition of fibrinogen in stage II therefore reduced the mean clotting time of plasma of the two groups of animals to a relatively comparable degree, 114 and 121 sec. respectively, but,

as a result of a deficiency in prothrombin and other factors, the mean stage-II clotting time of the diluted plasma of the treated animals was still approximately eight times that of the normal group.

The second bleeding from the treated animals was taken after the nine doses of choline, *dl*-methionine, or ethionine, or of the various vitamins, and the day following the second of two additional injections of carbon tetrachloride. In the choline group, two of three and in the vitamin E group one of two animals had stage-II clotting times shorter than any of those encountered in the carbon tetrachloride controls. In the methionine group, the stage-II clotting time values for three animals were 107, 151, and 167 sec. as compared with a minimum value of 253 sec. for the treated controls; the fourth animal had the very high value of 835 sec. All of the animals receiving vitamins K or B_{12} showed stage-I and stage-II clotting times as long as or definitely longer than these of the treated controls.

Third bleedings collected from all surviving animals after a rest period of 13 days, had the stage-I and stage-II clotting times within or close to the normal range (Table I).

Experiment 2

The first bleedings taken after nine doses of choline, methionine, or ethionine, and two or three days subsequent to the third injection of carbon tetrachloride had abnormally long clotting times in all three tests. *Ac*-globulin (labile factor), as well as fibrinogen and prothrombin, appeared to have been

TABLE I

THE EFFECT OF FEEDING VARIOUS SUPPLEMENTS ON THE PROLONGATION OF STAGE-I AND STAGE-II CLOTTING TIME INDUCED BY THE PREVIOUS AND CONCOMITANT INJECTION OF CARBON TETRACHLORIDE

Group	Supplement	Bleeding	No. tested	Clotting time (sec.)			
				Stage I		Stage II	
				Range	Mean	Range	Mean
Untreated	None	2	6	121–185	158	23– 41	31
Treated	"	2	3	335–508	419	253–359	300
	Choline	2	4	300–545	393	109–435	232
	Methionine	2	3	262–815	453	107–835	327
	Vitamin E	2	2	407–635	521	176–343	259
	" K	2	4	375–850	646	245–785	554
	" B_{12}	2	4	349–828	629	289–835	603
Untreated	None	3	6	117–169	148	17– 31	29
Treated	"	3	3	181–239	206	25– 42	31
	Choline	3	2	136–190	163	29– 39	34
	Methionine	3	2	198–205	201	23 –30	26
	Vitamin K	3	3	172–201	183	38– 44	42
	" B_{12}	3	3	175–211	196	19– 27	24

TABLE II

THE EFFECT OF CHOLINE, METHIONINE, AND ETHIONINE SUPPLEMENTS ON THE PROLONGATION OF CLOTTING TIME PRODUCED BY CARBON TETRACHLORIDE

Group	Supplement	No. tested	Clotting time (sec.)								
			Stage I			Stage II			Stage II + P.F.S.		
			Range	Mean	σ	Range	Mean	σ	Range	Mean	σ
Untreated	None	10	158– 222	180	23	24– 57	40	9.9	19– 39	31	5
Treated	None	10	329– 908	520	175	138– 765	412	206	113– 392	220	89
	Choline	10	243– 363	317*	35	63– 275	152*	70	68– 149	100	25
	Methionine	9	305–1000	605	123	165–1090	584	293	89– 648	269	167
	Ethionine	9	445–1075	837	250	226–1090	502	301	182–1075	328	286

* Significantly lower than mean values for ethionine control; $P_1 > 0{\cdot}001 < 0{\cdot}01$; $P_2 \cong 0{\cdot}001$.

reduced since the addition of prothrombin-free guinea pig serum very appreciably shortened the clotting time of the diluted plasma (Table II). Of the three supplements, only choline had any apparent effect in protecting against these changes in plasma coagulability and under the conditions of its administration prevented them only partially. The prolongation of coagulation time tended to be as great in the group of guinea pigs receiving the methionine supplement as in the carbon tetrachloride controls without supplement. The ethionine group had even more extended coagulation times.

The second bleeding taken the day following the 15th dose of these supplements and nine days after the third injection of carbon tetrachloride had almost normal clotting time values with the exception of those given ethionine. The third bleeding from the ethionine-treated animals, collected 17 days after the last injection of carbon tetrachloride and eight days after the last ethionine feeding, still showed delayed coagulability.

Effect on Complement Titer

Experiment 1

The five weekly injections of 0.2 ml. carbon tetrachloride resulted in a decrease in the mean complement titer of the 30 treated guinea pigs to 938 units per ml. as compared with a mean C′ titer of 1491 units per ml. for the six untreated controls (Table III). Titers of C′ 2 and C′ 3 were reduced but C′ 1 was not appreciably affected. A few animals showed a drop in C′ 4 titer; in the majority, this carbohydrate component of the complement complex did not deviate from the normal level.

The mean C′ titers of the second bleedings were not significantly different from those of the first bleedings (Table IV). Neither choline nor methionine feeding helped to restore complement titers in animals previously and

TABLE III

EFFECT OF FIVE, WEEKLY INJECTIONS OF CARBON TETRACHLORIDE ON THE COMPLEMENT TITER OF THE SERA OF GUINEA PIGS

Complement component	Group	Number tested	Estimated titer (units/ml.)		
			Range	Mean	σ
C′	Untreated	6	1190–1610	1491	176
	Treated	30	585–1110	938	186
C′ 1	Untreated	6	1670–5000	3160	1000
	Treated	30	2000–5000	3320	1030
C′ 2	Untreated	6	1250–2500	1968	683
	Treated	30	769–2500	1420	503
C′ 3	Untreated	6	1250–2000	1623	408
	Treated	30	416–2000	1285	852
C′ 4	Untreated	6	> 10,000	> 10,000	
	Treated	30	5000–> 10,000	> 9112	

TABLE IV

THE EFFECT OF FEEDING VARIOUS SUPPLEMENTS ON THE MEAN COMPLEMENT TITERS OF SERA OF GUINEA PIGS INJECTED WITH CARBON TETRACHLORIDE BEFORE AND DURING THE ADMINISTRATION OF THESE SUPPLEMENTS

Group	Bleeding	Supplement	Number tested	Mean titers (units/ml.)				
				C′	C′1	C′2	C′3	C′4
Untreated	2	None	6	1320	3000	2330	1750	>10,000
Treated	2	"	3	920	2430	1110	1110	>10,000
"	2	Choline	3	862	2670	1430	1110	>10,000
"	2	Methionine	4	914	2670	1250	1330	>10,000
"	2	Vitamin E	2	717	2050	1110	890	>10,000
"	2	" K	4	817	2470	806	830	7800
"	2	" B_{12}	4	564	2250	714	810	4800

TABLE V

THE EFFECT OF FEEDING VARIOUS SUPPLEMENTS ON THE COMPLEMENT ACTIVITY OF THE SERA OF THE FIRST BLEEDING OF GUINEA PIGS INJECTED WITH CARBON TETRACHLORIDE SUBSEQUENTLY AND CONCURRENTLY

Group	Supplement	Number tested	Complement component	Estimated titer (units/ml.)		
				Range	Mean	σ
Untreated	None	9	C′	1250–2170	1712	275
Treated	"	10	"	385–1470	808	315
	Choline	10	"	555–1250	938	220
	Methionine	9	"	172–1250	891	309
	Ethionine	9	"	125– 400	234	120
Untreated	None	9	C′1	3330–5000	3739	1455
Treated	"	10	"	1250–3330	2760	788
	Choline	10	"	3330–5000	4296	697
	Methionine	9	"	1110–5000	3143	1470
	Ethionine	9	"	1430–3330	1836	625
Untreated	None	9	C′2	1110–3330	2207	882
Treated	"	10	"	667–2500	1425	535
	Choline	10	"	1110–2000	1500	262
	Methionine	9	"	500–3330	1720	849
	Ethionine	9	"	286– 714	437	173
Untreated	None	9	C′3	1110–3330	1909	644
Treated	"	10	"	625–2000	1233	515
	Choline	10	"	1110–2220	1529	361
	Methionine	9	"	909–2500	1657	622
	Ethionine	9	"	167– 555	285	132
Untreated	None	9	C′4	10,000–>10,000	>10,000	—
Treated	"	10	"	2500–<10,000	<6050	—
	Choline	10	"	2500–<10,000	<7067	—
	Methionine	9	"	3330–10,000	7037	2865
	Ethionine	9	"	1110– 6670	3769	2288

concurrently under weekly treatment with carbon tetrachloride, while in the vitamin K and B_{12} groups, the mean C′ titers of 717 and 564 units per ml. were lower than the mean titers of the first bleeding from the same guinea pigs had been: 989 and 813 units per ml. The C′ 1 and C′ 3 titers remained relatively unchanged but the C′ 2 titers fell from mean values of 1648 and 1590 to 806 and 714 units per ml.

By the third bleeding taken after a rest period of 13 days, the complement titers of all surviving guinea pigs had been restored to essentially normal levels.

Experiment 2

The supplements choline and methionine were likewise ineffective in preventing the fall in C′ titer when they were fed to guinea pigs before and during the weekly injection of carbon tetrachloride (Table V). The mean titers of the various complements for sera from animals receiving the two lipotropic agents were not significantly higher than those of the carbon tetrachloride controls. Ethionine, moreover, had a marked reducing effect on whole complement titer and on C′ 4 as well as the three protein components, C′ 1, C′ 2, and C′ 3. In the four ethionine controls, which had not been injected with carbon tetrachloride, a similar although much less striking decline in all complement components except C′ 1 was recorded; the mean C′, C′ 1, C′ 2, C′ 3, and C′ 4 titers were 1291, 2708, 959, 1403, and 6251 units per ml. respectively.

On cessation of carbon tetrachloride treatment the complement titers returned rapidly to normal in the groups without supplement or with choline or methionine. The recovery was slower in all animals fed ethionine either alone or with carbon tetrachloride.

Discussion

Although considerable variation was observed in the reactions of individual animals, it seems justifiable to conclude that choline chloride, and to a lesser degree methionine, partially prevented the accumulation of fat in the livers of guinea pigs injected with carbon tetrachloride, probably through an accelerated conversion of the mobilized fat into phospholipids. That they also aided in maintaining the coagulative elements of the plasma, more particularly fibrinogen and *Ac*-globulin, may have been the secondary effect of their lipotropic action on the liver, or the two agents may also have acted as precursors, possibly supplying methyl groups, at some essential stage or stages in protein synthesis that was being blocked by the carbon tetrachloride. The fact that the protective effect of choline and methionine on the complement components was relatively slight would suggest that there may be fundamental differences in the action of carbon tetrachloride upon the synthesis of various blood proteins. Although the components of the coagulative and complement systems make up only a very small proportion of the total serum proteins, they are distributed throughout all major fractions with the exception of the albumins. For example, fibrinogen is precipitated in fraction I,

prothrombin and first component of complement in fraction III, and C′ 2 with the α-globulins in fraction IV of plasma (8, 11). C′ 3 has been shown to migrate electrophoretically with the β-globulins (17).

Titrations of complement and coagulative activities therefore may have certain advantages over other methods of detecting the effects of various agents and dietary conditions on serum proteins not only in view of their greater sensitivity than the usual chemical methods but because they reflect changes over a wider protein spectrum than such other highly sensitive methods as enzymatic determinations. The fact that first component of complement, C′ 1, was affected relatively little by carbon tetrachloride in our present series of experiments, whereas prothrombin was definitely depressed, indicates however that no general conclusions can be drawn in regard to the effect of a particular drug upon any one group of globulins.

Acknowledgments

The technical assistance of Misses Caroline Boulter and Elaine Bonfield is gratefully acknowledged.

References

1. Bach, S. J. The metabolism of protein constituents in the mammalian body. Oxford University Press, London. 1952. pp. 173-228.
2. Barret, H. M., Best, C. H., MacLean, D. L., and Ridout, J. H. J. Physiol. (London), 97 : 103. 1939.
3. Barret, H. M., Best, C. H., and Ridout, J. H. J. Physiol. (London), 93 : 367. 1938.
4. Bernheim, F. and Bernheim, M. L. C. Am. J. Physiol. 121 : 55. 1938.
5. Best, C. H. Federation Proc. 9 : 506. 1950.
6. Bier, O. G., Leyton, G., Mayer, M., and Heidelberger, M. J. Exptl. Med. 81 : 449. 1945.
7. Boulanger, P. and Rice, C. E. Can. J. Comp. Med. Vet. Sci. In press.
8. Cohn, E. J., Oncley, J. L., Strong, L. E., Hughes, W. L., Jr., and Armstrong, S. H., Jr. J. Clin. Invest. 23 : 417. 1944.
9. Dubnoff, J. W. Arch. Biochem. 22 : 474. 1949.
10. Du Vigneaud, V., Chandler, J. P., Cohn, M., and Brown, G. B. J. Biol. Chem. 134 : 787. 1940.
11. Edsall, J. T., Ferry, R. M., and Armstrong, S. H., Jr. J. Clin. Invest. 23 : 557. 1944.
12. Farber, E., Koch-Weser, D., Szanto, P. B., and Popper, H. Arch. Path. 51 : 399. 1951.
13. Farber, E., Simpson, M. V., and Tarver, H. J. Biol. Chem. 182 : 91. 1950.
14. Friedlander, H. D., Chaikoff, I. L., and Entenman, C. J. Biol. Chem. 158 : 231. 1945.
15. Handler, P. Proc. Soc. Exptl. Biol. Med. 70 : 70. 1949.
16. Haurowitz, F. Progress in biochemistry. Interscience Publishers, Inc., New York. 1950. pp. 100 and 138.
17. Jonsen, J., Manski, W., and Heidelberger, M. J. Immunol. 67 : 385. 1951.
18. Kibrick, A. C. and Blonstein, M. J. Biol. Chem. 176 : 983. 1948.
19. Koch-Weser, D., Farber, E., and Popper, H. Arch. Path. 51 : 498. 1951.
20. McHenry, E. W. and Patterson, J. M. Physiol. Rev. 24 : 128. 1944.
21. Rice, C. E., Boulanger, P., and Plummer, P. J. G. Can. J. Med. Sci. 29 : 48. 1951.
22. Rice, C. E., Boulanger, P., and Plummer, P. J. G. Can. J. Comp. Med. Vet. Sci. 16 : 216. 1952.
23. Rice, C. E., Boulanger, P., Plummer, P. J. G., and Annau, E. Can. J. Comp. Med. Vet. Sci. 16 : 230. 1952.
24. Rice, C. E., Boulanger, P., Plummer, P. J. G., and Annau, E. J. Immunol. 68 : 673. 1952.
25. Rice, C. E. and Crowson, C. N. J. Immunol. 65 : 201. 1950.
26. Stetten, D. J. Biol. Chem. 140 : 143. 1941.
27. Stetten, D. and Salcedo, J. J. Biol. Chem. 156 : 27. 1944.
28. Wahi, P. N. Arch Path. 47 : 119. 1949.
29. Ware, A. G. and Seegers, W. H. Am. J. Clin. Path. 19 : 471. 1949.

EFFECT OF PHENYLINDANEDIONE AND DICUMAROL IN EXPERIMENTAL FROSTBITE[1]

By Erica Lepp, Walter Chubaty, and Louis B. Jaques

Abstract

Rabbits and rats were exposed to frostbite and treated with the anticoagulants phenylindanedione and dicumarol. Phenylindanedione and dicumarol therapy did not prevent the formation of gangrene after frostbite, but did prolong the onset of gangrene in both rabbits and rats. Death from hemorrhage occurred after anticoagulant therapy in rabbits exposed to frostbite. Frostbite alone did not alter the prothrombin time of rabbits, but in rats it resulted in a significant increase in the prothrombin time. The rabbits showed a much greater increase in prothrombin time with the anticoagulants after frostbite.

Introduction

The question of the role of thrombosis, in the pathogenesis of gangrene following frostbite, has attained practical significance with the introduction of anticoagulants into clinical medicine. Lange and Boyd (5) observed that cold sufficient to solidify the exposed tissues causes a complete interruption of the circulation during the exposure. By means of the fluorescein test, they showed that this is always followed by a period of complete restoration of the circulation and increased capillary permeability. This period, which lasts for 6 to 16 hr. after exposure, is followed by one with arteriolar and capillary occlusion, due to the formation of red blood cell clots, which gives place to the onset of gangrene. Since the most severe exposure with solidification of tissue is always followed by a period of complete restoration of the circulation, heparinization subsequent to exposure seemed to Lange and Boyd (5), to be promising as a method of preventing the formation of clots. Their experiments showed that the exposed areas of the untreated animals developed gangrene, while the heparintreated group did not develop gangrene. This has been confirmed by Friedman, Lange, and Weiner (2). Lempke and Shumacker (7) have found heparin moderately effective in the treatment of frostbite. Other evidence that heparin assists in tissue repair has been presented by McCleery, Schaffarizich, and Light (8), who reported that the addition of heparin therapy to the treatment of a burn definitely speeded up the process of normal tissue repair and that the apparent sludging and congestion of the subjacent venous channels which occurred early in all animals seemed to recede much earlier in the heparinized series.

Theis, O'Connor, and Wahl (11) have observed that heparin plus dicumarol therapy given to patients with acute frostbite gave favorable results. However, Pichotka and Lewis (10) failed to confirm the results of Lange and Boyd,

[1] *Manuscript received January 20, 1953.*
Contribution from the Department of Physiology, University of Saskatchewan, Saskatoon, Sask. Supported by a grant-in-aid from the Defence Research Board of Canada.

and Hoelscher (4) reported that rabbits did not show any benefit from heparin treatment, so far as the extent and degree of necrosis of exposed legs were concerned. The death rate of heparinized animals was greater than that of nonheparinized frostbitten animals.

Experimental studies up to the present have been limited to those on the use of heparin. We have, therefore, studied the action of the indirect anticoagulants, phenylindanedione and dicumarol, on frostbite in rabbits and rats.

Methods

A total of 46 rabbits were subjected to frostbite by the method of Friedman, Lange, and Weiner (2). The rabbits each weighed 2–4 kgm. and were from the general laboratory mixed stock. Each rabbit was anesthetized with nembutal and a forefoot was depilated with a paste of barium sulphide – detergent – glycerol and covered with a thin rubber boot. In experiments where an ear was used, electric clippers were used to remove the hair from the ear and the ear was left uncovered. The foot or ear of the rabbit was placed in an alcohol–dry-ice bath at −30° C. to −40° C. for 20 to 30 min. It was then allowed to thaw at room temperature. The foot was then bathed in merthiolate, and covered with a sulphathiazole dressing, which was changed each day. No secondary infection was observed in the ears, which were therefore not treated with antibiotics. The circulation in the ears after frostbite was examined by the fluorescein method of Crimson and Fuhrman (1); 1.0 cc. of 5% fluorescein was injected intravenously, and the frozen ear was examined in ultraviolet light, at intervals up to two hours after the injection. The anticoagulants were given orally in gelatin capsules. Phenylindanedione (danilone) (100 mgm. per kgm.) was given initially, followed by 75 mgm. per kgm. per day in three divided doses for the duration of the experiment (until gangrene ensued or the animal died of hemorrhage). Dicumarol was given as a single dose of 5 mgm. per kgm. Anticoagulant therapy was started immediately after freezing. In preliminary experiments, the anticoagulants were given several days before frostbite so as to result in a pronounced prothrombinopenia at the time of freezing. This resulted in 63% mortality from hemorrhage in two days, so this procedure was discarded.

Frostbite was produced in rats by a method similar to one described by Dr. A. Blaustein in a private communication. Six series of 20 male rats, each weighing 150 gm. and placed in individual cages lined with newspaper, were put in the cold room at −15° C. to −25° C. until a stage of semiconsciousness was reached. After exposure to cold, the rats were given an initial dose of 100 mgm. per kgm. of phenylindanedione. This was then followed up by a maintenance dose of 30–40 mgm. per kgm. per day. The rats were given 10 gm. of ground food per day. The phenylindanedione was mixed with the food. Control groups were exposed to cold but not given phenylindanedione. Another control group was given the same dosage of phenylindanedione but was not exposed to cold.

A modified Quick procedure was used for determining the one-stage prothrombin time. The method of Herbert (3) was used to determine the two-stage prothrombin time. Blood from the rabbits was obtained from the ear vein and from the rats by cardiac puncture after exposure of the heart under ether.

Results

Effect of Phenylindanedione and Dicumarol on Incidence of Gangrene in Rabbits after Frostbite

Immediately after removal from the freezing mixture, the feet and ears were solid and grayish white in color. As thawing began the tissues became pink, and when thawing was complete, which was in about an hour, the tissues were bright red and swollen and remained that way for 7–18 hr. Edema developed proximal to the frozen part the day after freezing. Blisters also formed on the pads of the feet, and on the inner surface of the ear. The feet of control rabbits not on anticoagulant therapy turned dark red and cold within about three days, while the feet of treated rabbits remained warm and bright red at this time. The gangrene showed up first as blue brown spots, which later covered the whole frozen area. The tissue dried, and eventually fell off leaving a clean separation.

Thirty-nine rabbits were subjected to frostbite of one forefoot. Eighteen received phenylindanedione and 12, dicumarol. Seven rabbits were subjected to freezing of one ear, followed by phenylindanedione. The rabbits were inspected daily. In Table I is reported the average number of days for the first appearance of gangrene. However, a large number of animals receiving

TABLE I

EFFECT OF FROSTBITE AND ANTICOAGULANTS ON GANGRENE AND HEMORRHAGE IN RABBITS

(−30° C. to −40° C. for 20 min.)

	Number of animals	Gangrene		Hemorrhage	
		No. of cases	Days to onset	No. of cases	Days to death
Frostbite					
Control	9	9	3.1 ± 1.1	0	—
Phenylindanedione	18	11	6.4 ± 2.3	8	4.2 ± 1.6
	7*	4	5.2 ± 1.2	3	5.6 ± 1.5
Dicumarol	12	9	5.9 ± 2.8	3	4.0 ± 1.0
No frostbite					
Phenylindanedione	14	0	—	0	—
Dicumarol	13	0	—	0	—

* *One ear frozen; all others one forefoot frozen. One rabbit treated with phenylindanedione developed gangrene before it died of hemorrhage.*

NOTE: *S.d. as* ±.

anticoagulants (44%) developed hemorrhages and died before the onset of gangrene. We therefore recorded time to death for these animals and the average number of days to death is reported.

For rabbits not receiving anticoagulants, gangrene developed in 1–4 days (average 3.1 ± 1.1). For the rabbits receiving anticoagulants and not developing hemorrhage, gangrene developed 4–10 days after freezing (average for phenylindanedione 6.4 ± 2.3, for dicumarol 5.9 ± 2.8). It is evident therefore, that the anticoagulants delayed the onset of gangrene, but did not prevent it. On the fourth day, the treated animals showed a marked contrast to the untreated controls, the feet being completely healthy compared to the marked gangrene in the feet of the controls.

The Effect of Freezing on Prothrombin Time in Rabbits

The large number of animals showing hemorrhage in Table I was very surprising. In our experience, with the doses used, dicumarol rarely, and phenylindanedione hardly ever, results in hemorrhage in normal rabbits. Probably the high prothrombin time was involved in the hemorrhage, since death from hemorrhage occurred when the prothrombin time was high and since no hemorrhage occurred with freezing alone, but freezing was probably equally responsible as a precipitating factor. The site of hemorrhage was variable. Sometimes the frozen limb bled owing to the tearing off of the scab. At other times bleeding was from the mucous membrane of the nose and mouth, and the lungs were frequently congested.

To make a further study of the factors causing hemorrhage, four rabbits were given the routine dose of phenylindanedione for five days. The prothrombin time was determined daily. When the prothrombin time was back

TABLE II

EFFECT OF FROSTBITE WITH PHENYLINDANEDIONE ON PROTHROMBIN TIME IN RABBITS

Days on drug	Average proth. time ± s.d.	
	Drug	Drug + frostbite
0	10.4 ± 1.8	—
1	20.4 ± 1.0	18.7 ± 1.7
2	43.7 ± 2.9	36.4 ± 3.4
3	71.0 ± 1.2	63.6 ± 7.2
4	82.0 ± 6.6	220.0 ± 55.8
5	116.8 ± 4.9	310.0*
No. of deaths	0/4	3/4

* *No s.d. value for one rabbit.*

NOTE: *The drug was administered on day 0, immediately after determining the prothrombin time and freezing. The three rabbits died on the evening of the fourth day. The rabbit that survived developed gangrene.*

to normal, one forefoot of these rabbits was frozen by the method already described. The rabbits again received the routine dose of phenylindanedione for five days. As seen in Table II, no deaths occurred in this experiment from phenylindanedione treatment alone, but the same dose of phenylindanedione given to the same rabbits after frostbite resulted in the death of three out of four rabbits on the fourth day after freezing. Further, the prothrombin times when the drug was given after frostbite were significantly different. For the first three days, this was evident in a greater variability in values (greater s.d.). However, on the fourth day, the prothrombin time was increased threefold. In other experiments we have shown that with the same rabbit and same dose of phenylindanedione the prothrombin time response is quite reproducible.

It is evident that the prothrombin time in animals treated with phenylindanedione is affected by frostbite. Since the rabbits treated with the anticoagulant in Table I fell into two classes, those dying of hemorrhage and those surviving to develop gangrene, it was of interest to determine whether any connection could be established between prothrombin time response and clinical sequelae. Prothrombin times had been determined at intervals on a

TABLE III

EFFECT OF PHENYLINDANEDIONE WITH AND WITHOUT FROSTBITE ON PROTHROMBIN TIME OF RABBITS

Frostbite	Days after starting anticoagulant							Days to	
	0	1	2	3	4	5	6	Gangrene	Hemorrhage
One-stage prothrombin time (sec.)									
+	12.4 ±10.0 (3)	20.8 ±8.3 (4)	44.6 ±29.4 (8)	77.5 ±62 (6)	13.4 ±2.5 (3)	66.7 ±90 (6)	27.3 ±20.5 (4)	7.0 ±1.9 (9)	— — —
+	10.7 ±0.96 (4)	18.8 ±5.7 (4)	49.8 ±26 (6)	157.3 ±110 (8)	40.0 ±21 (2)	96.5 ±85 (2)	— — —	— —	5.3 ±1.1 (8)
−	14.1 ±1.7 (3)	20.0 *	20.2 ±9.8 (4)	22.9 ±13 (4)	28.0 ±9.7 (4)	22.0 ±12 (4)	19.5 ±5.5 (4)	No gangrene No hemorrhage (4)	
Two-stage prothrombin time (sec.)									
+	—	29.3 (3)	75.8 (7)	—	162.6 (6)	—	94.0 (3)		
−	—	31.7 (1)	73.2 (5)		107.4 (5)	—	30.0 (1)		

* *No s.d. value for one rabbit.*

NOTE: *Number of animals for the determination shown in parentheses.*

number of the rabbits described in Table I. The results are shown in Table III. Prothrombin times for the animals are shown together with the time to gangrene, and to hemorrhage. For this table the animals have been separated out and grouped according to whether they developed gangrene, or died of hemorrhage. It can be seen that after frostbite, the anticoagulant therapy resulted in much longer prothrombin times. However, there is no significant difference between the prothrombin time response of animals that later developed gangrene and animals that died of hemorrhage. On some of the animals the two-stage as well as the one-stage prothrombin time was determined. The two-stage prothrombin times are also shown in Table III. Both the one-stage and the two-stage methods show that, while there is considerable variation between rabbits with regard to the prothrombin time after a standard dose of dicumarol and phenylindanedione, this variation is much greater after frostbite. Thus on the fourth day of receiving phenylindanedione, normal animals showed prothrombin times of 16 to 39 sec., while animals on frostbite showed prothrombin times from 12 to 135 sec. That is, some animals seemed to show less resistance and others more resistance to phenylindanedione. This also appeared with dicumarol; there appeared to be no relation between the day of onset of gangrene and the prothrombin time response to phenylindanedione or dicumarol; likewise there seemed to be no relation between occurrence of hemorrhage and prothrombin time response to phenylindanedione or dicumarol.

Circulatory Changes After Frostbite

The fluorescein test of Lange and Boyd (5) was used to observe differences in the vascular changes following the phenylindanedione treatment. The tip of the rabbit's ear was immersed in an alcohol–dry-ice bath at −30° C. to −40° C. for 20 min. The ear was allowed to thaw at room temperature.

TABLE IV

AVERAGE DEGREE OF FLUORESCENCE IN FROZEN AREA OF RABBIT EAR AFTER PHENYLINDANEDIONE TREATMENT*

Time after fluorescein, min.	Number of days after freezing							
	1		2		3		4	
	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated
10	0±0	0±0	0.7±0.5	0±0	0.7±0.9	0±0.0	0.5±0.7	0
30	1.5±0.7	1.0±1.0	2.2±0.5	1.0±0	1.5±1.3	1.0±1.0	2.5±0.7	0
60	2.5±0.7	2.5±0.7	3.0	2.0	3.2±1.0	2.0±1.0	4.0±0.0	2.0
120	4.0±0	3.5±0.7	4.0±0	3.0±1.0	3.6±0.5	2.5±0.7	4.0±0.0	3.0

**1.0 cc. of 5% fluorescein injected intravenously and degree of fluorescence in ear recorded as: 4 +, frozen area complete fluorescent; 2 +, frozen area half fluorescent; 0, no fluorescence in frozen area.*

Where no s.d. is given the value is for a single observation.

Control animals not given frostbite show complete fluorescence of the ear 10 min. after the injection of fluorescein. This fluorescence declines and is almost completely gone after two hours.

Ten rabbits were treated in this way and fluorescein was injected from one to four days after freezing. The frozen ear was examined under ultraviolet light at intervals up to two hours after the dye injection. The degree of fluorescence in the frozen tip was recorded. In agreement with reports of Crimson and Fuhrman (1), the unfrozen part of the ear was fluorescent immediately after injection, while the frozen tip appeared black. Ten minutes after the fluorescein injection some of the rabbits receiving phenylindanedione showed fluorescent spots in the frozen tip, while the control rabbits not receiving the phenylindanedione treatment showed no sign of fluorescence in the frozen tip. Two hours after fluorescein the ears of all but one of the treated rabbits were completely fluorescent. One of the control rabbits showed complete fluorescence in the frozen ear after two hours, while the other controls were only partially fluorescent. These results are shown in Table IV.

As judged by this test the circulation in the exposed area for the first four days after freezing was better in the animals treated with phenylindanedione than in the controls.

Effect of Phenylindanedione on Frostbite in Rats

Frostbite was produced in rats placed in a cold room as described under methods. Immediately after the removal of the rats from the cold room, the toes and tails were solid and white, and the animals were in a state of semi-consciousness. After thawing, the frozen extremities became red, hot and edematous. In collecting data for this experiment results with the feet only were recorded. The first sign of gangrene was a blue brown color of the feet. The frozen tissue then dried and after about seven days fell off leaving a clean separation.

Twenty rats at a time (10 receiving phenylindanedione and 10 as controls) were given frostbite. A total of 120 rats were treated and the results are shown in Table V. After frostbite, rats not receiving phenylindanedione developed gangrene of the feet in 2.7 ± 0.6 days after frostbite. The animals not accounted for in this table were sacrificed for blood samples, before there

TABLE V

INCIDENCE OF GANGRENE AFTER FROSTBITE IN RATS

(−15° C. to −25° C.)

Treatment	Number of animals	Gangrene	
		Number of cases	Days to onset
Control	60	60	2.7 ± 0.6
Phenylindanedione	35*	33	5.8 ± 0.5

** 60 rats were given frostbite and phenylindanedione, 7 of these died of pneumonia and 20 were sacrificed for blood samples before the fifth day and are therefore not included in the total number of animals treated.*

TABLE VI

EFFECT OF FROSTBITE ON PROTHROMBIN TIME IN RATS (NO ANTICOAGULANT GIVEN)
PROTHROMBIN TIME, SEC. ± s.d.

Days	No. rats	Frostbite	Normal
1	5	20.3 ± 2.1	16.3 ± 1.1
3	5	19.0 ± 2.1	16.8 ± 0.8
5	5	22.4 ± 1.2	16.7 ± 0.3
7	5	25.8 ± 1.3	15.8 ± 0.6
10	10	30.4 ± 3.3	16.0 ± 2.1

was any sign of gangrene. The rats have not been separated out or grouped according to whether they developed gangrene or died of hemorrhage, since the early deaths in rats were due to pneumonia, before there was any sign of hemorrhage.

The effect of frostbite on the prothrombin time of rats was studied by exposing 30 rats to frostbite, sacrificing them at intervals, and determining the prothrombin time by the one-stage procedure. The results are shown in Table VI. A significant difference was found between the prothrombin time of nonfrostbitten and frostbitten rats. This suggests that frostbite has an effect on the clotting mechanism. The effect persisted as long as 10 days after frostbite.

Discussion and Conclusions

Treatment with either phenylindanedione or dicumarol in our hands was not successful in preventing the development of gangrene after frostbite but it did delay the onset of gangrene for about three days in both rabbits and rats. Lange and his co-workers have reported excellent results on the use of heparin in frostbite, but Pichotka and Lewis were unable to reproduce these results. Lange, Boyd, and Weiner (6) have suggested that these negative results obtained with heparin are due to a failure to maintain a high level of heparinization. This indicates that in Lange's series, a very high degree of hypocoagulability was used. Judging from the prothrombin times, the animals in the present series have a high degree of hypocoagulability.

Previous workers on the effect of anticoagulants in frostbite seem to have ignored the fact that a large percentage of their animals developed hemorrhage. Probably the most valuable information obtained in this study is the fact that the incidence of hemorrhage in animals on anticoagulant therapy is greatly increased after exposure to frostbite. Thus after frostbite, anticoagulant therapy resulted in the death of about 40% of the animals. No animals died as a result of anticoagulant therapy alone. On the basis of the results of Lange and others there appears to be no significant difference between the prothrombopenic agents and heparin in this regard. Up to date

the corresponding condition has not been observed in humans (9). This may possibly be due to the better control of anticoagulant therapy that can be achieved with a larger body mass or may be due to the fact that much lower dosages of these anticoagulants are used clinically compared to those used here to prevent gangrene.

In our experiments the effect of the anticoagulants on the prothrombin time failed to distinguish between those animals that die of hemorrhage and those that survive. On examining Table III it can be seen that there is no significant difference between the prothrombin time of the two groups.

The exposure to frostbite alone did not have any effect on the prothrombin time of rabbits. The rats on the other hand showed a significant increase in prothrombin time after frostbite. Rabbits exposed to cold showed a decreased resistance to anticoagulants. Thus anticoagulant therapy after frostbite resulted in an increase in the number of deaths due to hemorrhage and an increase in the prothrombin time on the fourth day (Table II). This difference in prothrombin time response to anticoagulants after frostbite might be due to the effect of the frostbite specifically or to the effect of a stress agent. In rats the effect of frostbite on the response to anticoagulant therapy is probably lost in the effect of frostbite itself on the prothrombin time.

Acknowledgments

We are indebted to Miss Mary Ogilvie for assistance in the preliminary investigations, and to Charles E. Frosst and Company for a generous supply of phenylindanedione (danilone) and dicumarol.

References

1. Crimson, J. M. and Fuhrman, F. A. J. Clin. Invest. 26 : 259. 1947.
2. Friedman, N. B., Lange, K., and Weiner, D. Am. J. Med. Sci. 213 : 61. 1947.
3. Herbert, E. K. Biochem. J. 34 : 1554. 1940.
4. Hoelscher, B. Arch. Path. 52 : 378. 1951.
5. Lange, K. and Boyd, L. J. Surg. Gynecol. Obstet. 80 : 346. 1945.
6. Lange, K., Boyd, L. J., and Weiner, D. Proc. Soc. Exptl. Biol. Med. 74 : 1. 1950.
7. Lempke, R. E. and Shumacker, H. B. Yale J. Biol. and Med. 21 : 321. 1949.
8. McCleery, R. S., Schaffarizich, W. R., and Light, R. A. Surgery, 26 : 548. 1949.
9. Orr, K. D. and Fainer, D. C. Medicine, 31 : 177. 1952.
10. Pichotka, J. and Lewis, R. B. Proc. Soc. Exptl. Biol. Med. 72 : 130. 1949.
11. Theis, F. V., O'Connor, W. R., and Wahl, F. J. J. Am. Med. Assoc. 146 : 992. 1951.

THE ABSORPTION OF LIGHT, IN THE VISIBLE AND ULTRAVIOLET, BY CERTAIN STEROIDS IN SULPHURIC ACID AND ETHANOL SOLUTIONS[1]

By J. H. Linford and O. Jacqueline Fleming

Abstract

The absorption spectra in the visible and ultraviolet regions of 17 steroids in sulphuric acid solutions have now been measured. The results in the visible region obtained on a further 10 steroids, listed in the present paper, confirm that colored compounds are formed in concentrated sulphuric acid at 57° C. only if an –OH group is present. Ketone groups contribute to the absorption only in the presence of an –OH group. Progressive addition of ethanol to the concentrated acid solutions of the steroids studied reveals a correlation of the visible absorption peaks with chemical groupings within the steroid molecule as follows: 255×10^2 cm.$^{-1}$, 240×10^2 cm.$^{-1}$, and 205×10^2 cm.$^{-1}$ to 210×10^2 cm.$^{-1}$ indicate an alcoholic C_3–OH; 220×10^2 cm.$^{-1}$ and 195×10^2 cm.$^{-1}$ indicate a phenolic C_3–OH; 220×10^2 cm.$^{-1}$ and 167×10^2 cm.$^{-1}$ to 175×10^2 cm.$^{-1}$ indicate ketone groups; and there is some evidence that 230×10^2 cm.$^{-1}$ and 205×10^2 cm.$^{-1}$ indicate an alcoholic group at the C_{17} position or in the C_{17} side chain. The inverse is not generally true because in several cases groups were not indicated by the absorption measurements; in the case of the estrogenic steroids estrone, estriol, and α-estradiol the absorption is characteristic of one grouping only. The absorption maxima in the ultraviolet region are not associated with particular chemical groups, but are a property of the steroid molecule. When certain maxima are arranged in a sequence of increasing wave numbers, the steroids fall into a chemical sequence.

Introduction

Previous studies (2 and 3) of seven steroid compounds have shown that hydroxyl and keto groupings in the steroid molecule, in acid–alcohol solution, can be detected by the positions of the visible absorption maxima. This paper describes similar absorption measurements, in the visible region, that have been made on an additional 10 steroids. In addition, the absorption measurements on the total of 17 steroids have been extended into the ultraviolet region, under the same solvent conditions as were used for the measurements in the visible region.

Procedure and Results

Visible Region

The spectrometric nomenclature and the methods of recording the results have been described in the earlier papers. The steroid nomenclature is that used in clinical practice, but where confusion may occur the name proposed by Fieser and Fieser has been added in brackets when a steroid is first mentioned.

The steroids examined were: β-estradiol (estradiol-17α), estrone, estriol, pregnanedione (pregnane-3,20-dione), androsterone, isoandrosterone (epiandrosterone), and desoxycorticosterone, supplied by the Ciba Company; and ergosterol, cholic acid, desoxycholic acid, and dehydrocholic acid obtained

[1] *Manuscript received March 6, 1952, and, as revised, February 23, 1953.*
Contribution from the Radiation Laboratories, The Manitoba Cancer Relief and Research Institute, Winnipeg, Manitoba.

from The Matheson Company. The steroids were used without further purification. A weighed amount of each steroid was dissolved in absolute ethanol and aliquot portions of these solutions were evaporated on a water bath to obtain the required amounts of the crystalline form.

Absorption measurements are stated in terms of D, the optical density, where $D = \log_{10} I_0/I$. The cell thickness was 1.00 cm. The spectral regions are measured in wave numbers (cm.$^{-1}$). Wave numbers are the more fundamental analytical measurement, as they express the energy differences in a particular molecule, which give rise to its absorption and fluorescence properties.

Series of reaction rate measurements were first carried out at 57° C. The reactions resulting from the addition of concentrated sulphuric acid to each crystalline steroid were followed by determinations of the absorption curves at measured intervals of time. In Fig. 1 the density value at a fixed wave number in the highest absorption maximum of each steroid is plotted against the time. Except in the case of estriol, a maximum of absorption is reached in one and one-half hours, and only relatively slow changes occur thereafter.

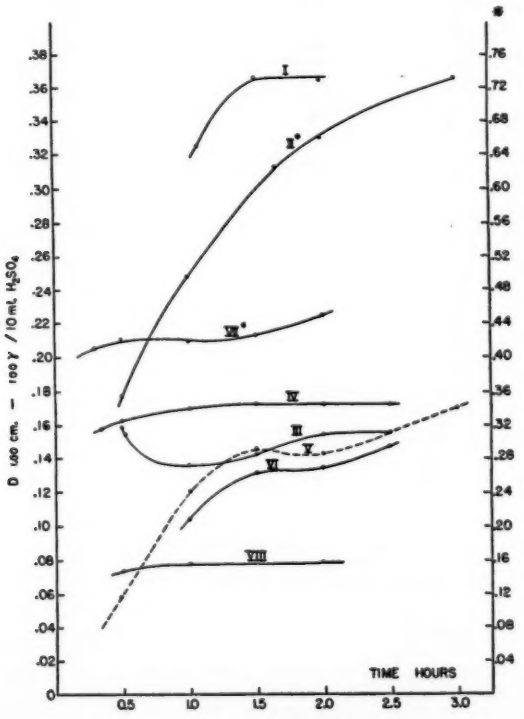


FIG. 1. Reaction rate curves of 100 γ of steroid dissolved in 10.0 ml. sulphuric acid at 57° C. I, estrone; II, estriol*; III, β-estradiol; IV, ergosterol; V, androsterone; VI, isoandrosterone; VII, cholic acid*; VIII, desoxycholic acid.
* Refers to the right hand, ordinate scale.

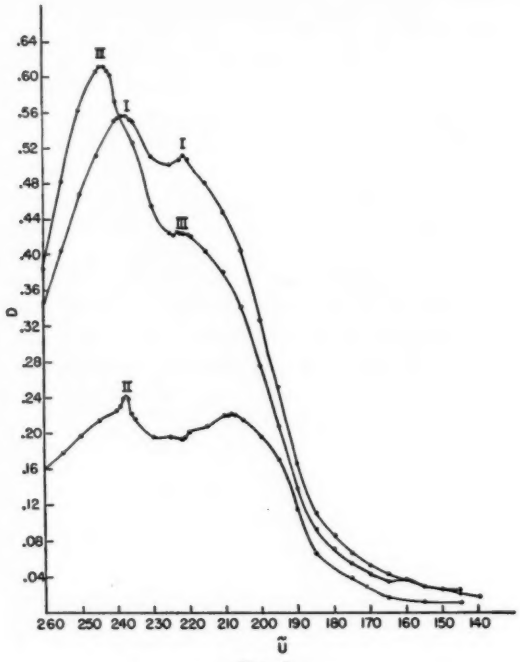


FIG. 2

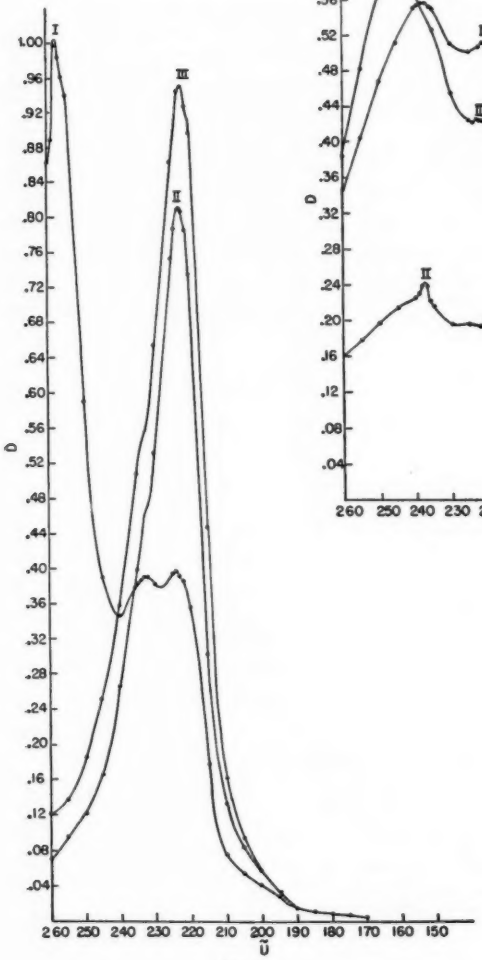


FIG. 3

FIG. 2. Absorption curves of steroids in sulphuric acid. I, 234γ androsterone in 6.0 ml. after 2.0 hr. at 57° C.; II, 234γ androsterone in 6.0 ml. after 0.5 hr. at 57° C.; III, 227γ isoandrosterone in 5.0 ml. after 2.0 hr. at 57° C. (Abscissa: wave numbers $\times 10^{-2}$; ordinate: optical density of 1 cm.)

Fig. 3. Absorption curves of steroids in sulphuric acid after 2.0 hr. at 57° C. I, 135γ β-estradiol in 4.0 ml.; II, 222γ estrone in 10.0 ml.; III, 174γ estriol in 8.0 ml. (Abscissa: wave numbers $\times 10^{-2}$; ordinate: optical density of 1 cm.)

The absorption curves of each steroid in sulphuric acid after having been heated for the stated time at 57° C. are shown in Figs. 2, 3, and 4. In the course of the reaction to this point no change of shape of the characteristic absorption curve is evident for estrone, estriol, ergosterol, cholic acid, and desoxycholic acid; they possess one major absorption peak. β-estradiol, androsterone, and isoandrosterone possess two absorption maxima of the same order of magnitude and during the first hour the ratios and positions of the two peaks alter appreciably. An example of this change of curve shape is shown in the case of androsterone in Fig. 2.

To prepare the acid–ethanol solutions, each steroid was dissolved in concentrated sulphuric acid at 57° C. for the time required for the absorption to reach the first maximum, as determined from Fig. 1; estriol was heated for two hours. The acid solutions were then cooled in ice-water and diluted

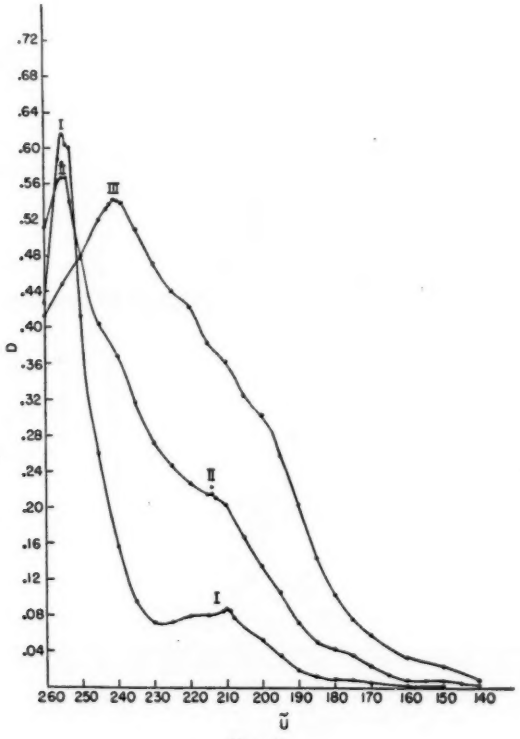


FIG. 4

FIG. 4. Absorption curves of steroids in sulphuric acid. I, 144γ cholic acid in 10.0 ml. after 0.5 hr. at 57° C.; II, 268γ desoxycholic acid in 4.0 ml. after 1.5 hr. at 57° C.; III, 256γ ergosterol in 8.0 ml. after 1.0 hr. at 57° C. (Abscissa: wave numbers $\times 10^{-3}$; ordinate: optical density of 1 cm.)

first with acid, then with ethanol. In this manner three solutions of the same concentration were obtained for each steroid, the solvents being 75, 50, and 25% sulphuric acid in absolute ethanol. The absorption curves of these solutions are plotted in Figs. 5–12.

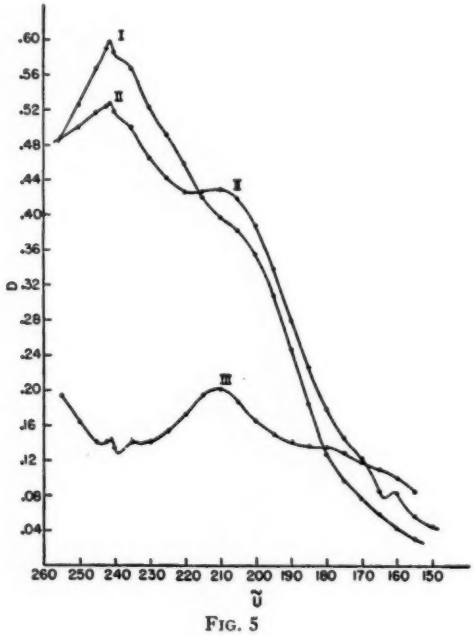


FIG. 5

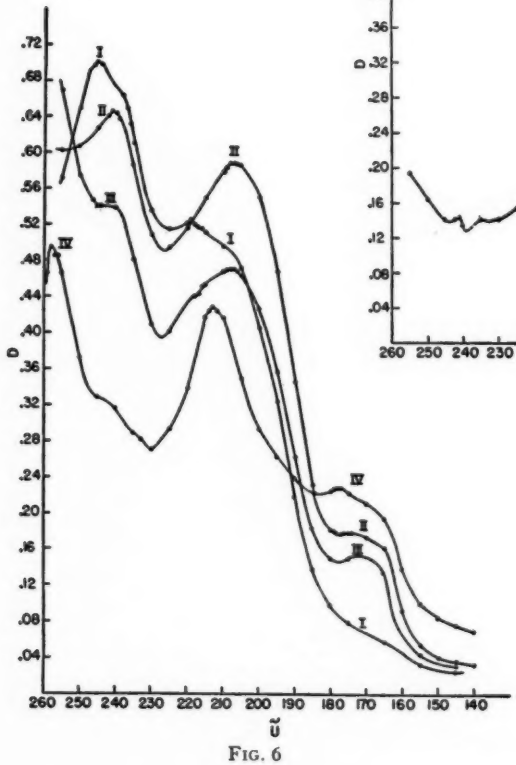


FIG. 6

FIG. 5. 256γ Ergosterol in acid, diluted to 8.0 ml.: I, 75%; II, 50%; and III, 25% sulphuric acid in ethanol. (Abscissa: wave numbers × 10^{-3}; ordinate: optical density of 1 cm.)

FIG. 6. 227γ isoandrosterone in acid, diluted to 5.0 ml. I, 75%; II, 50%; III, 42%; IV, 25% sulphuric acid in ethanol. (Abscissa: wave numbers × 10^{-3}; ordinate: optical density of 1 cm.)

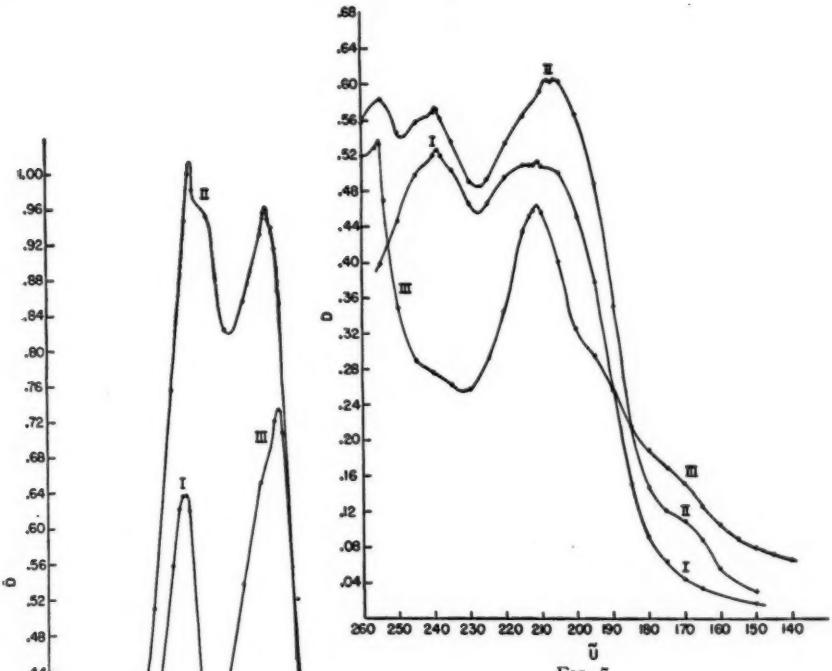


FIG. 7

FIG. 8

FIG. 7. 234γ androsterone in acid, diluted to 6.0 ml. of: I, 75%; II, 50%; III, 25% sulphuric acid in ethanol. (Abscissa: wave numbers $\times 10^{-2}$; ordinate: optical density of 1 cm.)

FIG. 8. 110γ estrone in acid, diluted to 10.0 ml. of: I, 75%; II, 50%; III, 25% sulphuric acid in ethanol. (Abscissa: wave numbers $\times 10^{-2}$; ordinate: optical density of 1 cm.)

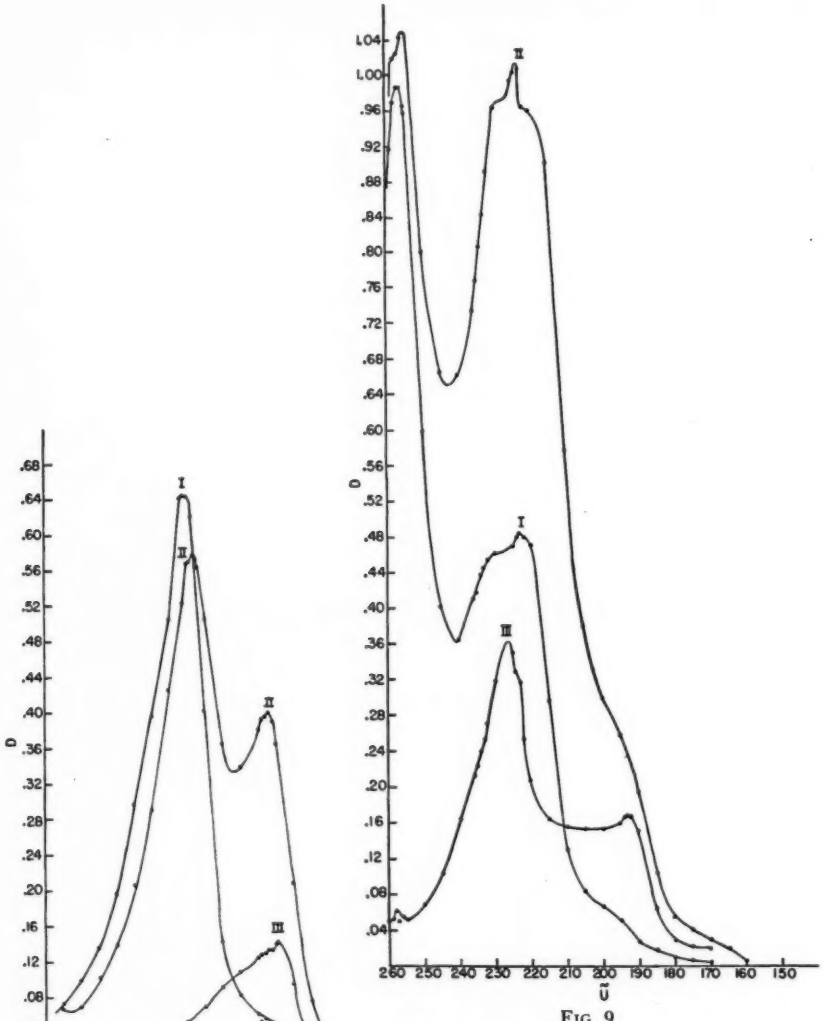


FIG. 9

FIG. 10

FIG. 9. 135γ β-estradiol in acid, diluted to 4.0 ml. of: I, 75%; II, 50%; III, 25% sulphuric acid in ethanol. (Abscissa: wave numbers $\times 10^{-2}$; ordinate: optical density of 1 cm.)

FIG. 10. 87γ estriol in acid, diluted to 8.0 ml. of: I, 75%; II, 50%; III, 25% sulphuric acid in ethanol. (Abscissa: wave numbers $\times 10^{-2}$; ordinate: optical density of 1 cm.)

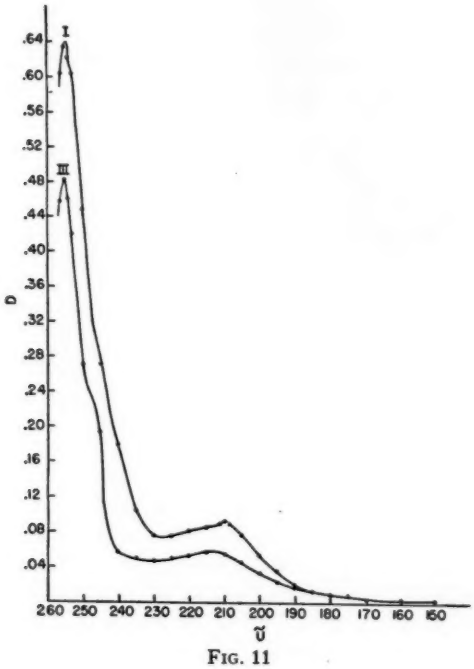


FIG. 11

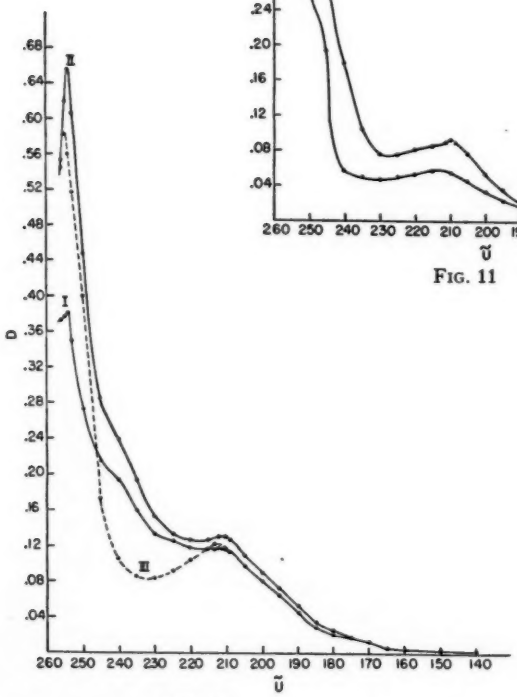


FIG. 12

FIG. 11. 144 γ cholic acid in acid, diluted to 10.0 ml. of: I, 75%; III, 25% sulphuric acid in ethanol. Results of the 50% acid are identical with I. (Abscissa: wave numbers $\times 10^{-2}$; ordinate: optical density of 1 cm.)

FIG. 12. 268 γ desoxycholic acid in acid, diluted to 8.0 ml. of: I, 75%; II, 50%; III, 25% sulphuric acid in ethanol. (Abscissa: wave numbers $\times 10^{-2}$; ordinate: optical density of 1 cm.)

TABLE I

OPTICAL DENSITIES (D) AND WAVE NUMBER POSITIONS ($\times 10^{-2}$ CM.$^{-1}$) OF THE ABSORPTION MAXIMA OF 100 γ OF STEROID

Steroid	Dissolved in 10.0 ml. concentrated sulphuric acid at 57° C.						Dissolved in 5.0 ml. sulphuric acid (57° C.); 5.0 ml. ethanol added at 0° C.					
	$\times 10^{-2}$ cm.$^{-1}$	D	$\times 10^{-2}$ cm.$^{-1}$	D	$\times 10^{-2}$ cm.$^{-1}$	D	$\times 10^{-2}$ cm.$^{-1}$	D	$\times 10^{-2}$ cm.$^{-1}$	D	$\times 10^{-2}$ cm.$^{-1}$	D
Cholic acid	350	.065	263	.459	253	.618	342	.086			255	.646
Desoxycholic acid	327	.221					360	.103	310	.100	262	.411
Dehydrocholic acid	450	.074	280	.006	257	.008	352	.017			255	.009
Cholesterol	450	.126	318	.226					323	.140		
Pregnanediol (pregnane-3α, 20α-diol)	420	.135	310	.285	300	.288	350	.080	325	.088		
Pregnanedione			325	.057					325	.041		
Isoandrosterone			323	.296					330	.165		
Dehydroisoandrosterone			327	.370					332	.208	327	.206
Androsterone	445	.119	323	.218					335	.142		
Testosterone			338	.755			450	.212	342	.384	270	.178
Progesterone	445	.187	343	.490					342	.462		
Desoxycorticosterone	445	.206	346	.557	268	.257			347	.366	270	.141
Estrone	440	.349	335	.542					350	.272	285	.108
Estriol	435	.421	327	.395			453	.545	352	.260		
β-Estradiol					275	.878			355	.118	272	.582
α-Estradiol	370	.254	338	.251			420	.490	370	.410		

Absorption measurements in the visible and ultraviolet regions on α-estradiol, estrone, and testosterone showed that Beer's Law holds in acid–ethanol solvents immediately after the addition of the alcohol to the concentrated sulphuric acid solutions of the steroids. Within the following hour the absorption of the stronger solution may become up to 15% higher than that calculated from measurements on the weaker solution.

Ultraviolet Region

The absorption measurements were made with an ultraviolet photoelectric spectrophotometer constructed in these laboratories and were checked with a Hilger Medium Quartz Spectrograph and Eastman type IV-0 plates, sensitized to the ultraviolet. The acid solutions for spectrometric examination were prepared by adding concentrated sulphuric acid to the crystalline steroids in test tubes and heating at 57° C. as described for the measurements in the visible region. Absorption measurements were made on this solution and on a second solution prepared by diluting the steroid in concentrated acid with an equal volume of absolute ethanol, while cooling in ice water. The measurements on the second solution were made immediately after the ethanol was added.

The positions of the absorption maxima within the spectral range from 250×10^2 cm.$^{-1}$ to 460×10^2 cm.$^{-1}$, and their densities calculated to represent 100γ of steroid in 10.0 ml. of the solvent, are listed in Table I.

The absorption maxima in the ultraviolet region of desoxycorticosterone in the acid and acid–alcohol solutions agree exactly in wave number with those of desoxycorticosterone acetate. The absorption density of the ester is 80% of the pure form when corrected for the difference in molecular weight.

Discussion

Visible Region

The results of the absorption measurements in the visible region confirmed and amplified those previously reported on seven steroids in sulphuric acid – ethanol solution, which are included in the following discussion.

No color was formed in concentrated sulphuric acid at 57° C. unless the steroid contained an –OH group; the solutions of progesterone, dehydrocholic acid, and pregnanedione were colorless. When the acid solution of pregnanedione was diluted with ethanol, a maximum of low density appeared at 255×10^2 cm^{-1}. Progressive dilution of the concentrated acid solutions of the sterols with alcohol resolved a series of absorption maxima at wave number positions associated with the presence of –OH and keto groups within the steroid molecule as follows:

At 50% acid concentration, the six neutral steroids possessing an –OH group at the C_3 position in a saturated *A* ring showed absorption maxima at 240×10^2 cm.$^{-1}$; additional maxima appeared at 250×10^2 cm.$^{-1}$ and 255×10^2 cm.$^{-1}$ for isoandrosterone and androsterone. At 25% acid concentration, the 240×10^2 cm.$^{-1}$ maxima of this group of steroids have been replaced by

maxima at 255 × 10^2 cm^{-1}. The persistence of maxima at 255 × 10^2 $cm.^{-1}$ for the bile acids when maxima at 240 × 10^2 $cm.^{-1}$ might be expected is therefore not an exception to the behavior of the alcoholic C_3 –OH group.

α-Estradiol, β-estradiol, estriol, and testosterone possess C_{17} –OH groups. In the first two cases absorption maxima at 230 × 10^2 $cm.^{-1}$ became evident as the concentrated acid solutions were diluted to 75% and 50% in alcohol. The absorption properties of estriol conform to those associated with the presence of a phenolic C_3 –OH group only. A study of the development of color by testosterone at 28° C. in concentrated sulphuric acid has shown the transient appearance of a maximum at 230 × 10^2 $cm.^{-1}$ after 17 hr., which is later masked by the 220 × 10^2 $cm.^{-1}$ maximum.

Pregnanediol and desoxycorticosterone possess an –OH group in the C_{17} side chain. The pregnanediol absorption curve shows an inflection at 230 × 10^2 $cm.^{-1}$ in 75% and 50% acid solution. Desoxycorticosterone has a low maximum at 230 × 10^2 $cm.^{-1}$ in concentrated sulphuric acid solution, and in the 75% acid in ethanol solution.

Steroids containing an alcoholic –OH group at the C_3, the C_{17} position, or in a C_{17} side chain showed a secondary maximum at 205 × 10^2 $cm.^{-1}$ to 210 × 10^2 $cm.^{-1}$ as the acid concentration was reduced. This peak is most strongly pronounced in the case of testosterone and confirms the presence of a low 230 × 10^2 $cm.^{-1}$ absorption in the stronger acid solution. This secondary peak was not evident in the case of β-estradiol; it may be masked by the rise of the 197 × 10^2 $cm.^{-1}$ maximum (See Fig. 9).

Sterols containing a keto group showed an absorption maximum at 220 × 10^2 $cm.^{-1}$ in the strong acid solutions. As the acid concentration was decreased, the 220 × 10^2 $cm.^{-1}$ maximum disappeared and secondary maxima appeared at 167 × 10^2 $cm.^{-1}$ to 175 × 10^2 cm^{-1}. Estrone showed only the characteristic behavior of the phenolic C_3 –OH group. In the case of the saturated keto steroids, androsterone and isoandrosterone, the intensity of absorption at 167 × 10^2 $cm.^{-1}$ was less by a factor of 10, than the corresponding absorption, of the keto steroids containing a double bond in the molecule.

In strong acid solutions the phenolic steroids, α-estradiol, β-estradiol, estriol, and estrone showed an absorption maximum at 220 × 10^2 $cm.^{-1}$ As the acid concentration was decreased the latter three steroids developed absorption maxima at 197 × 10^2 $cm.^{-1}$ The secondary peak at 197 × 10^2 $cm.^{-1}$ distinguishes the phenol group from the keto group. α-Estradiol in strong acid solution shows the 220 × 10^2 $cm.^{-1}$ maximum; in diluted acid only the maxima characteristic of the C_{17} –OH group are evident (2).

The relationships between the groups within the steroid molecule and the absorption maxima which have appeared in the present studies when the solutions of the steroids in concentrated acid are progressively diluted with alcohol are summarized in Table II. The peaks that appear in the strong acid solutions are referred to as the "primary maxima"; in the case of the bile acids this assumes the initial presence of a 240 × 10^2 $cm.^{-1}$ maximum.

TABLE II

Chemical groupings	Primary maxima		Secondary maxima			
	$\times 10^{-2}$ cm.$^{-1}$	(Å)	$\times 10^{-2}$ cm.$^{-1}$	(Å)	$\times 10^{-2}$ cm.$^{-1}$	(Å)
Alcoholic C_3 –OH	240	(4167)	255	(3922)	205–210	(4878–4762)
Alcoholic C_{17} –OH	230	(4348)			205	(4878)
C_{17} side chain containing –OH group	230	(4348)			205	(4878)
Phenolic C_3 –OH	220	(4545)			195–197	(5128–5076)
Ketone C –O	220	(4545)			167–175	(5988–5714)

The appearance of specific absorption maxima therefore indicates the presence of specific groups but the inverse is not necessarily the case. Cases arose in the estrogens, in which the chemical groups showed only one characteristic absorption. α-Estradiol did not show the phenolic –OH behavior in diluted acid solutions; estrone did not show the characteristic keto group behavior; estriol did not show the characteristic C_{17} –OH behavior. These steroids each possess two groups associated with characteristic absorption and one group predominated.

The 3:2 relationship of the densities of the absorption maxima of cholic acid to desoxycholic acid in 50% acid solution (see Table I) is of interest in demonstrating the connection of color with the –OH groups and in showing how the concentrated acid solvent masks this quantitative relation. The C_3, C_7, and C_{12} –OH groups must then affect the absorption equally. At 260×10^2 cm.$^{-1}$ the sensitivity of the visible instrument is low. The ultraviolet measurements (graphs not shown) listed in Table I are accurate.

TABLE III

SPECTRAL POSITIONS AND OPTICAL DENSITIES OF THE ABSORPTION MAXIMA OF CERTAIN STEROLS DISSOLVED IN AN ALCOHOLIC ACID REAGENT BY ALLEN, HAYWARD, AND PINTO. DATA CALCULATED TO 100 γ OF STEROL IN 10.0 ML. OF SOLUTION

Sterol	Optical densities at wave numbers $\times 10^{-2}$ cm.$^{-1}$							
	310	256	213	211	208	197	183	167
Pregnanediol		0.46			0.40			
Dehydroisoandrosterone		0.14						0.71
Androsterone		1.28	0.66				0.42	
Androstenediol*		0.17		0.26				0.40
Testosterone			1.20					0.76
Desoxycorticosterone	0.15				0.15			0.67
Estrone						0.232		

* *The reaction conditions have produced an absorption peak at 167 $\times$ 10^2 cm.$^{-1}$ in the case of androstenediol, which contains no ketone group in the initial state. This may be due to the heating of the acid solution of sterol in the presence of alcohol.*

Absorption curves published by Allen, Hayward, and Pinto (1), from which the measurements listed in Table III have been derived, substantiate the conclusions drawn in Table II. These authors used 2.0 ml. of a reagent consisting of one volume of 90% ethanol added to four volumes of concentrated sulphuric acid. They heated the solution for 12 min. at 55° C. and added 3.0 ml. of 95% ethanol. Thus the final solvent was 32% acid.

Ultraviolet Region

The absorption maxima in the ultraviolet region, of the steroids in concentrated acid solutions and in 50% acid solutions, bear no relationship to the visible absorption. No association of absorption maxima with chemical groups within the molecule is evident in Table I. The positions of the ultraviolet absorption maxima of progesterone and pregnanedione are not altered by heating the initial concentrated acid solutions to 100° C. to produce visible absorption. In acid solution estrone and estriol both possess absorption maxima in the visible region at 220×10^2 cm.$^{-1}$ and 195×10^2 cm.$^{-1}$; in the ultraviolet region the wave number positions of the absorption maxima of the two steroids differ. In the case of α-estradiol (2) the ultraviolet absorption was unchanged in two different solvent conditions under which the visible absorption was characteristic of the $C_{17}-OH$ group and the C_3 –OH group respectively.

The absorption maxima in the ultraviolet region are different for each steroid–acid complex and serve to characterize the molecule as a whole. In Table I the steroids of animal origin are listed in a chemical order of acidic alcoholic, ketonic, and phenolic types; certain of the absorption peaks then follow an increasing sequence on the wave number scale (Column 9). The sequence between 330×10^2 cm.$^{-1}$ and 370×10^2 cm.$^{-1}$ comprises the steroids under examination known to possess hormonal activity.

Acknowledgments

The authors wish to thank P. A. Macdonald, Director of the Manitoba Cancer Institute, for indicating the use of radiation measurements in the development of methods of hormone assay and for the development of some of the optical equipment used in these researches. This laboratory wishes to express its appreciation to the Ciba Company of Canada for its sympathetic interest, and for the provision of the hormones used in this work. We would also express our grateful thanks to the Trustees of the War Fund of the Brewers and Hotelkeepers of Manitoba, for the financial assistance which enabled this work to be undertaken.

References

1. Allen, W. M., Hayward, S. J., and Pinto, A. J. Clin. Endocrinol. 10 : 54. 1950.
2. Linford, J. H. Can. J. Med. Sci. 30 : 199. 1952.
3. Linford, J. H. and Paulson, O. Bernice. Can. J. Med. Sci. 30 : 213. 1952.

ENZYME INHIBITION BY DERIVATIVES OF PHENOTHIAZINE

VI. INHIBITION OF GLYOXALASE ACTIVITY OF HUMAN AND RABBIT ERYTHROCYTES[1]

By H. B. Collier

WITH THE TECHNICAL ASSISTANCE OF

Sheila C. McRae

Abstract

Phenothiazone has been found to be a strong inhibitor of glyoxalase activity of human and rabbit erythrocytes. Concentrations for 50% inhibition were 10^{-6} *M* for intact cells and 10^{-4} *M* for haemolysates with added glutathione. Glyoxalase activity was also markedly inhibited by phenothiazine, methylene blue, and *p*-chloromercuribenzoate; slightly inhibited by alloxan and phenylhydrazine; and not affected by dialuric acid. Enzyme inhibition did not parallel methaemoglobin formation. The possible relationship of these findings to the haemolytic action of phenothiazine is discussed.

Previous papers in this series have described the inhibition of rat-liver succinoxidase by phenothiazine and by phenothiazone (4), as well as the inhibition of rat-brain hexokinase by phenothiazone (3). The activity of these enzymes may involve sulphydryl groups (17) and it is possible that the thiazines act through these sulphydryl groups. Since administration of phenothiazine may cause an acute haemolytic anaemia in some species (5, 18), it appeared desirable to test the effect of thiazines upon the sulphydryl enzymes in the mammalian erythrocyte.

Dakin and Dudley (7) in 1913 first showed the presence of glyoxalase in erythrocytes, and this red-cell enzyme has been further investigated by Jowett and Quastel (11), Jones and McCance (10), and Alivisatos and Denstedt (1). Lohmann (13) showed that reduced glutathione is the coenzyme of the glyoxalase system. Racker (16) and Crook and Law (6) have recently investigated the mechanism of action, and have shown that 'glyoxalase' is made up of two enzymes, one catalyzing the addition of glutathione to substrate, and the other catalyzing the hydrolysis of this addition compound. In the present investigation, no attempt has been made to distinguish between the two enzymes, and the inhibitions reported are those of the over-all 'glyoxalase' system.

Methods

Commercial phenothiazine was recrystallized from hexane, and this was used to prepare phenothiazone (phenothiazone-3) and the sulphoxide (phenothiazine-5-oxide) as previously described (4). Methylglyoxal (pyruvic aldehyde, 30% solution) was obtained from Bios Laboratories, Inc., and reduced glutathione was the product of Fisher Scientific Co.

[1] *Manuscript received February 4, 1953.*

Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alberta, with financial assistance from the Defence Research Board of Canada. (Grant No. DRB-352.)

Erythrocytes from human or rabbit blood were washed three times with 1% sodium chloride solution, and the cells were made up to 20 times their volume with the saline. Glyoxalase activity was determined on this 1 : 20 suspension of intact cells or upon a haemolysate prepared by adding water instead of saline. Determinations were carried out, in Summerson differential manometers at 37° C., by a modification of the manometric method of Jowett and Quastel (11).

For the determination of the activity of haemolysates the following were pipetted into the main vessels:—

0.30 ml. of erythrocyte suspension
1.10 ml. of water or solution of inhibitor
0.20 ml. of 0.20 *M* sodium bicarbonate
0.30 ml. of 0.020 *M* reduced glutathione.

Into the side-arms of the right-hand flasks was pipetted 0.20 ml. of approximately 0.10 *M* methylglyoxal (the 30% solution diluted 1 : 40 and neutralized with sodium bicarbonate), and into the side-arms of the left-hand flasks, 0.20 ml. of water. The final volumes on mixing were thus 2.00 ml. and the final concentrations were: sodium bicarbonate 0.020 *M*, glutathione 0.0020 *M*, and methylglyoxal, 0.010 *M*.

For measurement of the activity of intact erythrocytes, the 1.10 ml. of water was replaced by an equal volume of 1.1% sodium chloride, or of inhibitor dissolved in 1.1% sodium chloride, and the glutathione was omitted. In all cases, solutions of inhibitors were adjusted to pH 7.4, if necessary, before addition to the flasks.

The flasks were gassed for 10 min. with a mixture of 5% carbon dioxide – 95% nitrogen, and the contents of the side-arms were then tipped in. The manometer stopcocks were left open for two minutes after the mixing, after which they were closed, and readings were taken every five minutes for 20 min.

Preliminary experiments were done upon rabbit erythrocytes, which were obtained by heart puncture with heparin as the anticoagulant. Later experiments were performed on human blood which was taken into acid–citrate–dextrose solution (2 gm. disodium citrate monohydrate and 3 gm. anhydrous glucose made up to 100 ml.). This blood was kept in the refrigerator for not more than a week and glyoxalase activity remained virtually constant.

In the manometric determinations, the rate of carbon dioxide production was constant for the first 20 min., whether intact erythrocytes or haemolysates were employed. Hence, microliters of carbon dioxide produced in 20 min. at 37° C. was taken as the measure of glyoxalase activity. Under these conditions, the rate of carbon dioxide production was found to be exactly proportional to enzyme concentration, 0.30 ml. of 1 : 20 cell suspension liberating about 100 – 120 μl. of carbon dioxide. Haemolysates, with the added glutathione, gave about the same activity.

Results

Glyoxalase of Rabbit Erythrocytes

Effect of Phenothiazone

Preliminary experiments indicated that phenothiazone was a powerful inhibitor of glyoxalase. But it had been found that about one hour was required for maximum inhibition of rat-brain hexokinase by this compound (3), and therefore the effect of time upon the inhibition of glyoxalase was examined. Prolonging the time of contact between enzyme and inhibitor up to one or two hours had very little effect upon the degree of inhibition, whether of intact cells or of haemolysates. Hence, in subsequent experiments, the enzyme preparation and inhibitor were mixed just before the manometers were set up.

The concentration of phenothiazone was then varied; the results of typical experiments upon intact cells and upon haemolysates are represented in Fig. 1. The concentrations corresponding to 50% inhibition were: 10^{-4} *M* for haemolysates and 1.3×10^{-6} *M* for intact erythrocytes.

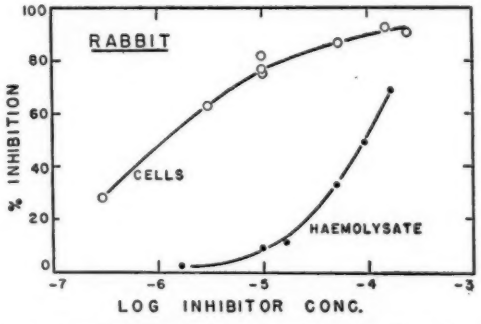


FIG. 1. Effect of phenothiazone concentration (expressed as the logarithm of the molarity) on the degree of inhibition of glyoxalase activity of rabbit erythrocytes.

When the concentration of glutathione that was added to haemolysates was varied, both the activity of the enzyme and the degree of inhibition by phenothiazone were altered. The results of such an experiment are given in Table I.

TABLE I

EFFECT OF GLUTATHIONE CONCENTRATION UPON GLYOXALASE ACTIVITY OF HAEMOLYSATES OF RABBIT ERYTHROCYTES

Concentration of added glutathione, *M*	Enzyme activity		Inhibition, %
	Control, μl. CO_2	Phenothiazone, 7×10^{-5} *M*, μl. CO_2	
0	8	10	—
0.0003	58	16	72
0.0033	115	80	30

Effects of Other Inhibitors

Table II summarizes the results obtained with a variety of other compounds. Both phenylhydrazine hydrochloride (recrystallized from ethanol) and *p*-chloromercuribenzoic acid were neutralized with equivalent quantities of base before use. Since phenothiazine is insoluble in water it was added as 0.1 ml. of alcoholic solution, and 0.1 ml. of ethanol was added to the control flasks. The recorded concentration of phenothiazine is based upon the amount added.

Visual observation of the brown color gave evidence of methaemoglobin formation in the presence of phenylhydrazine.

TABLE II

INHIBITION OF GLYOXALASE ACTIVITY OF RABBIT ERYTHROCYTES BY VARIOUS COMPOUNDS

Compound added	Concentration, *M*	Enzyme activity		Inhibition, %
		Control, μl. CO_2	Treated, μl. CO_2	
Intact cells				
Phenothiazine	5×10^{-5}	102	19	82
Phenothiazine sulphoxide	5×10^{-5}	102	51	50
Phenylhydrazine	1×10^{-3}	102	14	86
p-Chloromercuribenzoate	5×10^{-4}	102	12	88
Haemolysates				
Phenylhydrazine	1×10^{-3}	110	98	11
p-Chloromercuribenzoate	1×10^{-4}	110	108	2

GLYOXALASE OF HUMAN ERYTHROCYTES

Effect of Phenothiazone

Phenothiazone was observed to inhibit the glyoxalase activity of human erythrocytes, both intact cells and haemolysates. The results of a representative experiment with varying phenothiazone concentrations are presented in Fig. 2. The concentrations required for 50% inhibition were: 10^{-6} *M* for intact cells and 10^{-4} *M* for haemolysates, values that are virtually identical with those obtained for rabbit erythrocytes.

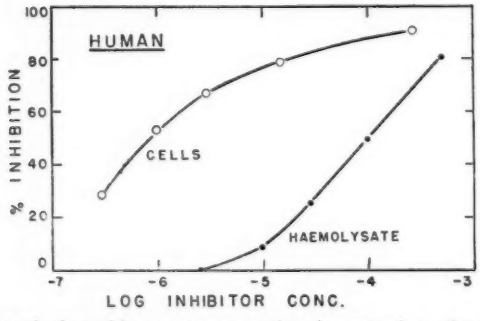


FIG. 2. Effect of phenothiazone concentration (expressed as the logarithm of the molarity) on the degree of inhibition of glyoxalase activity of human erythrocytes.

Addition of 0.002 *M* glutathione did not increase the glyoxalase activity of intact erythrocytes, but it did reduce somewhat the inhibition produced by phenothiazone. In the presence of 3×10^{-6} *M* phenothiazone, the degree of inhibition was decreased from 68% to 48% by addition of the glutathione.

Effect of Phenothiazine

Phenothiazine, added as 0.1 ml. of alcoholic solution, was found to inhibit the activity of both intact cells and haemolysates. In a typical experiment, the degree of inhibition for various amounts of added phenothiazine was as follows:

Intact cells
0.002 micromoles added — 21% inhibition
0.100 micromoles added — 84% inhibition

Haemolysates
0.010 micromoles added — No inhibition
0.100 micromoles added — 22% inhibition

In each case methaemoglobin formation was observed in the presence of 0.1 micromoles of added phenothiazine.

Effect of Other Inhibitors

Various other compounds were tested upon the glyoxalase activity of intact human erythrocytes, and the results are summarized in Table III. Phenylhydrazine hydrochloride and dialuric acid solutions were neutralized before addition. Of the compounds tested, only phenylhydrazine caused visible methaemoglobin formation in the cells.

TABLE III

EFFECT OF VARIOUS COMPOUNDS UPON THE GLYOXALASE ACTIVITY OF INTACT HUMAN ERYTHROCYTES

Compound	Concentration, *M*	Enzyme activity, μl. CO_2	Inhibition, %
Methylene blue	0	126	0
	10^{-6}	117	15
	10^{-5}	76	40
	10^{-4}	27	79
Phenylhydrazine	0	102	0
	10^{-4}	60	41
	10^{-3}	12	88
Alloxan	0	110	0
	10^{-4}	96	13
Dialuric acid	0	110	0
	10^{-4}	104	5

Activity of Stroma-free Haemolysates

Previous investigators apparently have not determined whether the glyoxalase activity of the erythrocytes is associated with the stroma or with the cell contents. Hence, a stroma-free preparation of human erythrocytes was made. Washed cells were haemolysed with 20 times their volume of 0.001 *M* acetic acid, which also precipitated the stromata or 'ghosts'. One portion of the haemolysate was centrifuged for 15 min. at 3000 r.p.m. to remove the 'ghosts'. The glyoxalase activities of 0.30 ml. volumes of the stroma-free haemolysate and the uncentrifuged haemolysate were then compared, with added glutathione in each case. The activities were 82 and 99 μl. of carbon dioxide for the stroma-free and the whole haemolysate respectively. It was concluded that most of the enzyme was present in the cell interior.

Discussion

It is concluded from these experiments that phenothiazone is a powerful inhibitor of the glyoxalase activity of human and rabbit erythrocytes. The enzyme of the intact erythrocytes is much more sensitive to the inhibitor than is the enzyme in haemolysates with added glutathione. This striking difference may be due to binding of the inhibitor by the cells. (We found that approximately one half of the phenothiazone was bound when a 1 : 20 suspension of human erythrocytes was added to an equal volume of 2.5×10^{-5} *M* phenothiazone.)

On the other hand, the enzyme in the intact erythrocytes is concentrated within a very small volume, whereas in haemolysates it is diluted to a much larger total volume. Hence the inhibition kinetics would not likely be identical, and this may account for the difference in the shape of the curves for intact cells and for haemolysates as given in Figs. 1 and 2.

It had been assumed that phenothiazone would act upon the sulphydryl groups of the enzyme, but this does not appear to explain the results obtained. Phenothiazone inhibits the enzyme in the presence of a large excess of glutathione, in contrast to *p*-chloromercuribenzoate which has no inhibitory action under these conditions. (Another difference is that the mercury derivative haemolyzes the cells while phenothiazone does not.) Also, alloxan, which reacts with sulphydryl groups, is not a powerful glyoxalase inhibitor. It is probable that binding of phenothiazone by the cell protein (2) is a factor in the inhibitory action.

Among the various inhibitors tested, no parallelism was found between methaemoglobin formation and glyoxalase inhibition. Phenothiazine forms methaemoglobin and phenothiazone does not, yet they are both powerful glyoxalase inhibitors. Methaemoglobin is readily formed in the presence of phenylhydrazine which is a weaker inhibitor.

Phenothiazine itself can probably act as an electron transfer agent, and its antioxidant properties have recently been described by Dam and co-workers

(8) and by Murphy, Ravner, and Smith (15). Michaelis, Granick, and Schubert (14) demonstrated the univalent oxidation of phenothiazine to a semiquinone free radical.

Various investigators have recently stressed the importance of sulphydryl compounds in maintaining the integrity of the erythrocyte. Alivisatos and Denstedt (1) have investigated the glyoxalase activity of stored whole blood. Fegler (9) has reported on a possible relationship between the reduced glutathione content and spontaneous haemolysis in horse blood. Kun (12) has suggested that thiols and glyoxalase serve to protect tissues against methylglyoxal intoxication.

Nevertheless, the present experiments cannot be said to throw any light on the actual haemolytic mechanism of phenothiazine and its derivatives.

References

1. Alivisatos, S. A. and Denstedt, O. F. Trans. Conf. preservation of formed elements and proteins of blood. 105. 1949.
2. Allenby, G. M. and Collier, H. B. Arch. Biochem. Biophys. 38 : 147. 1952.
3. Allenby, G. M. and Collier, H. B. Can. J. Med. Sci. 30 : 549. 1952.
4. Collier, H. B. and Allenby, G. M. Can. J. Med. Sci. 30 : 443. 1952.
5. Collier, H. B. and Mack, G. E., Jr. Can. J. Research, E, 22 : 1. 1944.
6. Crook, E. M. and Law, K. Biochem. J. 52 : 492. 1952.
7. Dakin, H. D. and Dudley, H. W. J. Biol. Chem. 14 : 423. 1913.
8. Dam, H., Kruse, I., Prange, I., and Søndergaard, E. Acta Physiol. Scand. 22 : 299. 1951.
9. Fegler, G. Nature, 170 : 624. 1952.
10. Jones, P. E. H. and McCance, R. A. Biochem. J. 45 : 464. 1949.
11. Jowett, M. and Quastel, J. H. Biochem. J. 27 : 486. 1933.
12. Kun, E. J. Biol. Chem. 187 : 289. 1950.
13. Lohmann, K. Biochem. Z. 254 : 332. 1932.
14. Michaelis, L., Granick, S., and Schubert, M. P. J. Am. Chem. Soc. 63 : 351. 1941.
15. Murphy, C. M., Ravner, H., and Smith, N. L. Ind. Eng. Chem. 42 : 2479. 1950.
16. Racker, E. J. Biol. Chem. 190 : 685. 1951.
17. Singer, T. P. Brewers Digest, 20 : 85, 94, 104, 109. 1945.
18. Swales, W. E., Collier, H. B., and Allen, D. Can. J. Research, D, 20 : 349. 1942.

PRESSURE AS A POSSIBLE CAUSE OF DISSOLUTION AND REDEPOSITION OF BONE AND TOOTH CRYSTALS[1]

By R. E. Glegg and C. P. Leblond

Abstract

Many workers have observed resorption of bone and tooth substance at the site of action of normal or artificially applied pressure. The mechanism by which pressure exerts this effect is not known, although it has been attributed to production of osteoclasts or to changes in circulation. An hypothesis, based on the physicochemical fact that pressure increases the solubility of crystals, is here presented to explain pressure-induced bone resorption. This hypothesis satisfactorily accounts for otherwise inexplicable results obtained in recent investigations on the distribution and turnover of radiophosphate in bone and tooth.

For almost a century, there have been claims that bony tissues may be resorbed under the influence of pressure (7, 13, 23). Possibly the most dramatic example is the erosion of the bone overlying an expanding aneurysm (1) or tumor (13, 20, 25). Under physiological conditions, pressure may induce the resorption of bony tissues, as for instance, when an erupting permanent tooth erodes most of the root of the superimposed deciduous tooth (16).

Erosion of bone and tooth has also been produced experimentally. Using histological techniques, Oppenheim observed that when a tooth was artificially pressed against its bony socket, resorption occurred (15). These experiments gave a sound scientific basis to the previous empirical use of pressure in orthodontia to displace teeth through the bone substance of mandible and maxilla (12). A different approach was used by Jores, who placed a load on a limited area of the vertebral column, and thus produced in the vertebral spines a histologically visible resorption, which increased with increased pressure (8).

These effects of pressure have generally been attributed to the production of osteoclasts or to changes in circulation (13). It is, however, suggested here that a direct physical effect of pressure on bone and tooth crystals causes their dissolution. Theoretical considerations will be presented in support of this hypothesis. It will then be shown that the hypothesis explains three complex cases arising out of recent investigations.

The Relationship Between Pressure and Solubility

Owing to the weight of the body, muscular action, and other factors, the crystals making up bone and tooth are subjected to pressure under normal physiological conditions. These crystals are surrounded by a fluid which, according to Logan and Taylor (11), appears to be saturated with respect to the ions making up the crystalline structures. Therefore, the laws governing the growth and dissolution of crystals in saturated solutions should apply to bones and teeth.

[1] *Manuscript received February 25, 1953.*
Contribution from the Department of Anatomy, McGill University, Montreal, Que.

In the second part of the nineteenth century, Thomson (21), Poynting (17), and Riecke (18) recognized the fundamental principle that a crystal under linear pressure has a greater solubility than one not under such pressure. As a consequence, a crystal subjected to pressure may dissolve even in a saturated solution. Recently, Correns and Steinborn (4, 5) formulated an equation describing the relationship between a crystal under pressure and a solution saturated with the ions composing the crystals:

$$P\,v = R\,T\ln(c/c_s)$$

where P = the linear pressure acting on the crystal,
v = the volume of a mole of the crystal substance,
R = the gas constant,
T = the absolute temperature,
c = the concentration of the solution when it reaches equilibrium with the crystal subjected to the pressure P,
c_s = the equilibrium concentration of the solution when the crystal is under no linear pressure, i.e., the "saturation concentration".

R is constant, and, if the temperature T is kept constant, c_s and v are also constants for a given crystal. If P is increased, c must therefore increase. In order to satisfy this condition, the crystal must dissolve.

In a simple system composed of pressed and unpressed crystals, the dissolution of the pressed crystals renders the solution supersaturated with respect to the crystals not subjected to the linear pressure. The resulting supersaturation is then reduced by growth of the unpressed crystals (and by formation of new crystal nuclei).

Bone is an even more complex system in which crystals may press against one another in various directions. Some understanding of the situation may be gained by use of the concepts developed in studies of geological structures, since some rocks, like bones, consist of accumulations of crystals in an intercrystalline fluid. Turner and Verhoogen (22) have stated that, if an external pressure is applied to such rocks, the pressure effectively received by each portion of the system will depend on the relative positions of the constituent crystals. At points of greatest pressure, dissolution occurs, a local supersaturation ensues, and at points of least pressure, redeposition of material takes place. There will thus be a tendency towards reorientation of the crystals so that the imposed pressure will become evenly distributed and, therefore, produce a minimal effect, thus leading to a more stable system.

An Interpretation of Three Complex Phenomena

1. The Decrease in Intensity of Localized Radioautographic Reactions at Growth sites

It was previously shown (10) that, after P^{32} injection, there were two types of radioautographic reaction in bone: (1) A *diffuse* type, due to the radioactive phosphate ions incorporated into the surface layer of the bone crystals by a process of exchange between the labeled phosphate ions of the interstitial fluid and the nonlabeled ions of the crystal surfaces. Since the blood level of P^{32} falls rapidly with time, the labeled ions in the crystal surfaces are subsequently replaced by exchange with the now unlabeled ions of the blood and extracellular fluid. As a consequence, the diffuse reactions decrease rapidly

with time. (2) A *localized* type limited to areas of growth, due to radioactive phosphate ions incorporated even into the deeper layers of the crystals as a result of accretion of new bone salts. The localized reactions were previously thought to persist indefinitely, but more precise observations in 50 gm. rats (9) indicated that these reactions decreased in intensity and spread out during the first week after injection, after which time there was no further appreciable change. (The decrease in intensity was more than could be accounted for by the loss of the labeled ions from the crystal surfaces by exchange as the blood level of P^{32} decreased.)

These observations led to three conclusions, namely, (*a*) since the localized radioautographic reactions were produced by crystals containing P^{32} in their deeper layers, the decrease in these reactions must have been due to the *dissolution* of some of these crystals to such an extent that the labeled, deeper layers were involved; (*b*) the spreading out of the remaining reactions pointed to the *redeposition* of some of the dissolved material on crystals located at some distance from those dissolving; and (*c*) the maintenance of the reactions for periods longer than a week after P^{32} injection indicated the *stability* of the persisting crystals.

These observations are consistent with an explanation derived from the concepts developed in the study of geological structures (22). As new crystals are laid down in growth areas of bone, some of them may be subjected to more pressure than others and will, therefore, dissolve. As a result, a local supersaturation will take place. This will be reduced partly by a loss of phosphate to the plasma (hence the decrease of radioautographic reactions) and partly by a redeposition on less pressed crystals (hence the spreading out of the reactions). As a result of the redeposition, the pressure will even out and the tendency to dissolve will be minimized (hence the indefinite persistence of reactions after the first week).[2]

2. *The Decrease in the Intensity of Incremential Dentine Bands at the Dentinoenamel Junction in Young Teeth*

Bélanger and Leblond (3) showed that after injection of radiophosphorus into very young animals there were, as in bone, diffuse and localized radioautographic reactions in the dentine. The diffuse reaction soon disappeared owing to exchange with the rapidly decreasing P^{32} of plasma. The localized reaction then became visible as a dark band which, at one day after injection, was limited to the inner portion of the dentine. Up to four days after injection, this band appeared to move peripherally as more and more dentinal substance was laid down on the inner surface of the dentine. At this early time, no apparent decrease in the intensity of the reaction band with time was observed. However, in an investigation carried out for longer periods of time after P^{32} injection, Bélanger (2) observed that the reaction band widened to some extent as it approached the outer portion of the dentine (dentinoenamel junction), where it gradually faded, leaving only a weak, widespread reaction.

[2] *Such an arrangement may result in the orientation of bone crystals (revealed by the polarization microscope).*

The decrease in the intensity of the reaction indicated that radioactive crystals of dentine were dissolving. This *dissolution* may again be due to the direct effect of pressure on the crystals. The accumulation of new dentine material on the concave side of the young tooth would exert a pressure directly opposed to that due to accumulation of new enamel material on the convex side. The predominance of the dissolution near the dentinoenamel junction may be attributed to local variations in pressure, possibly due to structural irregularities at the junction. Crystals subjected to a greater intensity of pressure would dissolve preferentially.

That there is *redeposition* of the dissolved material which has not been lost to the plasma is again indicated by the spreading out of the reaction.

3. The Decrease in the Mineral Content of Intact Bones in Animals with Fractures

Roche and Mourgue (19) found a moderate decrease in phosphate content not only in the fractured bone in rats, but also in the corresponding intact bone. This was apparent up to 21 days after fracture, after which time there was a return to normal values.

The decreased phosphorus content must have been the result of a *dissolution* of some of the crystals in the intact bone. This in turn may be due to the increased pressure which the animals bring to bear on the intact limb owing to disuse of the fractured limb. In addition, the direction of the applied pressure in the intact limb may have changed, and thus the crystals may have dissolved at *new* points of high pressure.

Redeposition of material on less pressed crystals must also have occurred here to correct the supersaturation resulting from the dissolution of crystals under pressure. This is indicated by observations of Wilkinson and Leblond (26), who showed that in rats, the intact bone contralateral to a fractured bone took up a much larger amount of P^{32} than the corresponding bone in normal animals without fracture. This larger uptake must have consisted of extracellular phosphate which jointly with the material dissolved from the pressed crystals was deposited on the less pressed crystals.

In conclusion, it is suggested that the three cases quoted above are explained by a direct effect of pressure inducing dissolution of bone or tooth crystals.[3]

[3] *In addition to forces pressing bone or tooth, it is necessary to consider forces acting in the opposite direction, that is, exerting a pull on bone or tooth. Such forces, referred to as "tensions" have been shown by Weidenreich (24) to produce bone growth, and he cites instances in which an abnormally high tension induced ossification in tendons and ligaments. The best known case is that of the displacement of teeth in jaws, where the "pressed" area in front of the advancing tooth dissolves, while the "pulled" area behind the tooth grows (15). Earlier, Wolff (27) on the basis of results of Volkmann (23) and Hüter (7), had clearly stated that pressure produces bone resorption, while tension produces bone growth.*

At the crystal level, tension may produce growth, since tension may be considered as a negative pressure, and from the above equation, such a pressure would tend to decrease c, that is, to produce a departure of ions from the solution onto the crystals, thus resulting in crystal growth.

The internal stresses due to the action of a force on an object consist of both pressures and tensions, which extend in the various parts of the object according to the recognized laws of mechanics (13). Thus, a single force acting on a bone might produce pressure with crystal dissolution in some areas, or tension with crystal growth in other areas of the same bone. Such dual response to forces like body weight, muscular contraction, organ growth, etc., may play a role in the development of long bones, skull, etc. (Incidentally, this fact might explain why under certain conditions pressure may appear to produce growth (13)).

This dissolution is followed by the redeposition of part of the dissolved material on less pressed crystals.[4] *Thus, in general, the action of pressure on bones or teeth would produce dissolution of crystals at points bearing increased pressure, followed by redeposition of material elsewhere in the neighborhood.* These phenomena may occur as a result of pressures generated under physiological conditions and thus may play a role in the development of bones and teeth.

Acknowledgment

This work was supported by a grant from the Defence Research Board, Department of National Defence (Canada).

References

1. ANDERSON, W. A. B. Pathology. The C. V. Mosby Company, St. Louis. 1948. Fig. 423.
2. BÉLANGER, L. F. Anat. Record, 114 : 529. 1952.
3. BÉLANGER, L. F. and LEBLOND, C. P. Proc. Soc. Exptl. Biol. Med. 73 : 390. 1950.
4. CORRENS, C. W. Discussions Faraday Soc. 5 : 267. 1949.
5. CORRENS, C. W. and STEINBORN, W. Z. Krist. Mineral. Petrog. Abt. A, 101 : 117. 1939.
6. HEVESY, G. Radioactive indicators. Interscience Publishers, Inc., New York. 1948.
7. HÜTER, C. Arch. klin. Med. 25 : 572. 1862; 26 : 484. 1863.
8. JORES, L. Beitr. path. Anat. u. allgem. Path. 66 : 433. 1920.
9 LEBLOND, C. P., KUMAMOTO, Y., and GLEGG, R. E. Unpublished results.
10. LEBLOND, C. P., WILKINSON, G. W., BÉLANGER, L. F., and ROBICHON, J. Am. J. Anat. 86 : 289. 1950.
11. LOGAN, H. A. and TAYLOR, H. L. J. Biol. Chem. 119 : 293. 1937.
12. MCCOY, J. D. Applied orthodontia. Lea and Febiger, Philadelphia. 1927. p. 161 and following.
13. MURRAY, P. D. F. Bones. Cambridge University Press, London. 1936.
14. NEUMAN, W. F. and MULRYAN, B. J. J. Biol. Chem. 185 : 705. 1950.
15. OPPENHEIM, A. Angle Orthodontist, 5 : 159, 233. 1935; 6 : 5, 69, 153. 1936.
16. ORBAN, B. Dental histology and embryology. P. Blakiston's Son & Co., Philadelphia. 1929. Figs. 162 and 163.
17. POYNTING, J. H. Phil. Mag. Ser. 5, 12 : 32. 1881.
18. RIECKE, A. Nachr. Ges. Wiss. Göttingen. Math.-physik. Klasse, Fachgruppen. 278. 1894.
19. ROCHE, J. and MOURGUE, M. Bull. soc. chim. biol. 21 : 143. 1939.
20. SARNAT, B. G. and SCHOUR, I. Oral and facial cancer. The Year Book Publishers Inc. Chicago, Ill. 1950. p. 115.
21. THOMSON, J. Phil. Mag. Ser. 4, 24 : 395. 1862.
22. TURNER, F. J. and VERHOOGEN, J. Igneous and metamorphic petrology. McGraw-Hill Book Company, Inc., New York. 1951. p. 523.
23. VOLKMANN, R. VON. *Quoted by* Wolff (see Reference 27).
24. WEIDENREICH, F. Z. Anat. Entwicklungsgeschichte, 69 : 558. 1923.
25. WEINMANN, J. P. and SICHER, H. Bone and bones. The C. V. Mosby Company, St. Louis. 1947. p. 357 and following.
26. WILKINSON, G. W. and LEBLOND, C. P. Surg. Genecol. Obstet. In press.
27. WOLFF, J. Das Gesetz der Transformation der Knochen. A. Hirschwald. 1892.

[4] *Whether or not the dissolution and redeposition of crystalline material described in the present work are similar to the phenomena described under the name of "recrystallization" by Hevesy (6) and especially by Neuman and Mulryan (14) remains to be decided.*

POLIOMYELITIS IN CANADIAN ESKIMOS

LABORATORY STUDIES. IV. ANTIGENIC TYPING OF VIRUS STRAINS IN MONKEYS AND IN TISSUE CULTURES[1]

By W. Wood[2], Eina M. Clark[2], F. T. Shimada[2], and A. J. Rhodes[2]

Abstract

Three strains of poliomyelitis virus isolated from Eskimos infected in the outbreak of poliomyelitis occurring at Chesterfield Inlet, N.W.T., in February 1949 have been typed. This typing has been carried out in monkeys and in tissue cultures. Typing was carried out by preparing in rhesus monkeys antiserum to the Eskimo strains and testing these for capacity to neutralize a fixed dose of the Brunhilde (Type 1) and Leon (Type 3) strains on thalamic inoculation of monkeys, and of the Lansing (Type 2) strain on cerebral injection of mice. Antisera prepared against Strains E3-56 and E3-227 completely neutralized Brunhilde virus, as shown on thalamic inoculation of virus–serum mixtures in groups of five monkeys. In contrast, these sera did not neutralize Leon virus. Tests carried out in mice showed that antisera to these Eskimo strains failed to neutralize Lansing virus. It was concluded therefore that Eskimo Strains E3-56 and E3-227 belong to Type 1. The same two sera were tested for their capacity to inhibit the cytopathogenic effect induced in roller-tube cultures of monkey testis by representative strains of the three types (Brunhilde, Y-SK, and Saukett). The two Eskimo sera inhibited the cytopathogenic effect induced by the Brunhilde strain but not the changes induced by the Y-SK and Saukett strains, confirming that the corresponding Eskimo viruses belonged to Type 1. Eskimo Strains E3-79 and E3-227 have also been typed in tissue cultures by a direct method; in this method antisera to the three prototype viruses were tested for capacity to inhibit the cytopathogenic effect induced by the Eskimo strains. Results of this test were in agreement, the Eskimo strains belonging to Type 1. Two strains of virus isolated from the Chesterfield Inlet outbreak have previously been typed by U.S. investigators, by techniques involving the use of monkeys. A total of five strains isolated from this epidemic have therefore been typed, and all have been found to belong to Type 1. This study illustrates that the typing of poliomyelitis strains can be carried out more rapidly and more economically in tissue culture than in monkeys.

Introduction

The epidemic of poliomyelitis that occurred at the settlement of Chesterfield Inlet, N.W.T., in the winter of 1948–49 proved of much interest to workers in the field of poliomyelitis, because of the occurrence in mid-winter and the high rate of attack on a susceptible population group. The clinical and laboratory features of the outbreak have been described by Adamson *et al.* (1) and Peart (13). We have previously reported the isolation of typical poliomyelitis viruses from pathological specimens obtained from six Eskimos (3, 14). This outbreak aroused our interest in the general immunology of poliomyelitis in Eskimos, for it appeared that these people had considerably less exposure to poliomyelitis viruses than almost any other group in the world. To investigate the degree of such previous exposure to poliomyelitis viruses, sera collected at the time of the Chesterfield Inlet outbreak were tested for the presence of antibody to Type 2 (Lansing) poliomyelitis virus; this antibody was found in the serum of 18 of the 34 Eskimos studied, and was

[1] *Manuscript received February 16, 1953.*
Contribution from Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario. Aided by a Public Health Research Grant (Government of Canada).
[2] *Connaught Medical Research Laboratories.*

demonstrated in the serum of children aged 10 and over (3). Because of the inaccessibility of Chesterfield Inlet, we have been unable to continue our investigations into the immunology of poliomyelitis in Eskimos residing in this area. However, we were able to obtain the sera of 99 Eskimos from another remote section of the Northwest Territories, Pangnirtung, on the eastern shore of Baffin Island. Type 2 antibody was found in only 10 of these samples, all from persons aged 18 and over (4). There is no record of poliomyelitis ever having occurred in Pangnirtung or vicinity. Findings in these Pangnirtung Eskimos were broadly similar to those reported in the extensive series of observations on Type 2 antibody levels in north Alaskan Eskimos by Paul and his associates (10, 11, 12).

Because of the general interest of the Chesterfield Inlet outbreak, we considered it advisable to proceed with the typing of the strains of virus isolated. Two strains were sent for examination by The Committee on Typing of the National Foundation for Infantile Paralysis. These two strains (E3-213, E3-117) have now been reported as belonging to Type 1, the common North American Type (2, 21). Three further strains have since been typed in our laboratory by techniques involving the use of monkeys and tissue cultures, and these results are reported in this paper.

Until recently it was possible to type poliomyelitis viruses only in monkeys; in the study of the Committee on Typing of the National Foundation for Infantile Paralysis 100 strains were so typed (5). In one of the methods used by these investigators, rhesus monkeys were vaccinated with the prototype strains (Brunhilde, Lansing, or Leon), and later "challenged" intracerebrally with the virus to be typed. This method is only satisfactory if the virus to be typed regularly produces paralysis in monkeys, and if a satisfactory 50% paralyzing titer (PD_{50}) can be calculated. In the second method, antisera were prepared to the prototype viruses by vaccination of rhesus monkeys. The virus to be typed was then tested by the virus neutralizing technique with these standard sera. In this method also the virus strain to be typed has first to be adapted to monkeys and then titrated. In the third and more economical method developed by Salk *et al.* (20), immune serum was prepared by vaccinating monkeys with the untyped strain, the preparation of virus being mixed with Freund's paraffin oil adjuvants to increase antigenicity. The immune serum was then tested for neutralizing activity against standard suspensions of the three prototype viruses. The method was particularly applicable to the typing of Strain E3-177 which titered only $10^{-2.0}$ in monkeys (7).

During the last two years, alternate methods of typing poliomyelitis viruses in tissue culture have been developed, and these depend on the fact that poliomyelitis immune serum will specificially inhibit the degenerative ("cytopathogenic") changes that poliomyelitis viruses produce in cells in tissue culture (8, 17, 18, 24, 25, 26). These tests can be carried out by the original technique of Enders in which fragments of tissue are suspended in nutrient fluid in small Erlenmeyer flasks and infected with virus, for immune serum

will specifically inhibit virus propagation. In the method which is now more popular, roller-tube cultures of fibroblasts are infected with mixtures of virus and type-specific antisera. No cytopathogenic changes will be observed in fibroblasts in the cultures containing the mixture of virus and homotypic immune serum. In contrast, those cultures that contain virus and heterotypic sera will show cytopathogenic changes.

These methods are suitable for strains of poliomyelitis virus that produce cytopathogenic changes in tissue cultures, but some strains do not produce a marked cytopathogenic effect, for example, the prototype Lansing strain itself. To overcome this difficulty, it has been recommended that such strains be typed by the method of Salk in which monkeys are immunized with the untyped virus; the immune serum is then employed in tests with virus strains belonging to the three types that produce well-marked cytopathogenic changes (16).

When typing is being carried out by the method involving the inhibition of the cytopathogenic changes induced by the untyped virus, it is usual to employ antisera prepared in monkeys immunized with the three prototype viruses (Brunhilde, Lansing, Leon). When typing is carried out with antiserum prepared to the untyped virus it is then necessary to use strains belonging to the three types that produce a well-marked cytopathogenic change; various strains have been so recommended, for example, for Type 1, the Brunhilde and Mahoney, for Type 2, Y-SK and MEF1, and for Type 3, the Leon and Saukett strains.

Part I : Typing in Monkeys

Materials and Methods

Adaptation of Eskimo Strains to Monkeys

Three strains of virus isolated from Eskimos at Chesterfield Inlet were selected for typing (E3-56, E3-79, and E3-227). In order to have available "pools" of tissue representing these strains, it was first necessary to passage the viruses by the cerebral route in rhesus monkeys. The results of inoculating rhesus monkeys thalamically with the strains are shown in Table I. Each strain was passed three times through rhesus monkeys. It was observed that the strains were not highly pathogenic, for in the second and third passages only about 50% of the animals developed paralysis, although most of the remaining monkeys became infected, as shown histologically. Pools of 20% suspension of spinal cord enlargements were prepared from monkeys developing paralysis at the third passage level. It was not necessary to titrate these pools in rhesus monkeys. The method of preparation of these pools follows that used in previous studies (15).

Prototype Strains Used in Typing Studies

In order to type the three Eskimo strains it was necessary to have pools representative of the three types of poliomyelitis virus. The representative

TABLE I

RESULTS OF PASSAGING ESKIMO STRAINS OF POLIOMYELITIS VIRUS IN RHESUS MONKEYS

Reference number of strain	Passages through monkeys*									Number of monkeys used to furnish tissue for pool
	First passage			Second passage			Third passage			
	Inoculum	Monkeys paralyzed	Monkeys positive histologically	Inoculum	Monkeys paralyzed	Monkeys positive histologically	Inoculum	Monkeys paralyzed	Monkeys positive histologically	
E3-56	Human brain and cord suspension	2/2	2/2	Suspension of monkey spinal cord enlargements	1/4	2/4	Suspension of monkey spinal cord enlargements	4/10	8/10	4
E3-79	Stool (ultracentrifuged)	1/1	1/1	Suspension of monkey spinal cord enlargements	2/4	2/4	Suspension of monkey spinal cord enlargements	4/10	9/10	4
E3-227	Throat washings	2/2	2/2	Suspension of monkey spinal cord enlargements	1/2	1/2	Suspension of monkey spinal cord enlargements	5/11	8/11	5

* *Inoculum of 0.8 ml. injected intrathalamically.*

strains were those used by the Committee on Typing of the National Foundation for Infantile Paralysis as prototypes, namely the Brunhilde (Type 1), Lansing (Type 2), and Leon strains (Type 3). The details are given in Table II.

Preparation of Antiserum in Monkeys

The pools of the three Eskimo strains of virus were incorporated with Freund's paraffin-oil adjuvants as previously described (15). Rhesus monkeys were then immunized by injections given intramuscularly every two weeks over a period of 12 weeks. Blood was taken from the animals at the start of the program (week 0) and at weeks 4, 8, and 12. The sera representing the bleedings made 12 weeks from the start of the vaccination program were pooled, and these serum pools served as the antisera to the Eskimo strains. The details of the immunization program are shown in Table III. The sera were titrated in tissue culture as described below, but despite the prolonged course of immunization, antibody titers did not rise higher than $10^{-2.0}$, a level substantially lower than we have found in animals immunized with other strains of virus.

Virus Neutralization Tests with Prototype Viruses and Eskimo Antisera

Undiluted pooled Eskimo monkey antisera were mixed with a sufficient amount of previously titrated Brunhilde monkey pool to give a final amount of virus in the virus–serum mixture of 100 PD_{50}. After the mixtures had stood at room temperature for one and one-half hours, they were injected thalamically (0.8 ml. amounts) into five rhesus monkeys. The monkeys were killed at the first sign of paralysis. Animals which did not become paralyzed were killed four weeks after inoculation. All animals were examined histologically, and the final diagnosis rested on these results. The tests with Leon virus were carried out in monkeys in a precisely similar manner. In order to

TABLE II

DETAILS OF POOLS OF PROTOTYPE POLIOMYELITIS VIRUSES

Name of strain	Details of pool		
	Source of tissue	Passage level since original isolation	50% end point
Brunhilde	Monkey central nervous system	6	$10^{-5.2}$*
Lansing	Monkey central nervous system	Approximately 210	$10^{-4.2}$†
Leon	Monkey central nervous system	4	$10^{-6.0}$*

* *Titrations carried out by thalamic inoculation of rhesus monkeys (0.8 ml.) with serial dilutions of pool; four monkeys used per dilution.*

† *Titer determined by inoculation of groups of 10 mice with serial dilutions of pool.*

TABLE III

IMMUNIZATION OF MONKEYS WITH ESKIMO VIRUSES

Immunizing strain	Ref. Nos. of monkeys immunized	Injection of virus at following weeks*	Monkeys bled at following weeks	Titer of serum pools prepared from week 12 bleedings†
E3-56	1003 1004 1005			$10^{-2.0}$
E3-79	1007 1008 1009	0, 2, 4, 6, 8, and 12	0, 4, 8, and 12	$10^{-1.0}$
E3-227	1010 1012 1013			$10^{-2.0}$

** Monkeys received intramuscular injections of 0.3 gm. cord material mixed with adjuvant at weeks 0 and 2; 0.2 gm. cord and adjuvant were given in subsequent weeks.*

† Titers of serum determined by inhibition of cytopathogenic effect produced by homologous virus in roller-tube tissue cultures.

economize on the use of monkeys, the tests for Lansing antibody were carried out by the inoculation of virus–serum mixtures cerebrally (0.03 ml.) into groups of 12-14 gm. male mice. Sera obtained from individual monkeys at weeks 4, 8, and 12 of the immunization program were tested individually for Lansing antibody in mice.

Results

Type 2 Virus

The results of inoculating mice with mixtures of Lansing virus and Eskimo antisera are shown in Table IV, from which it will be seen that the sera did not neutralize Lansing virus. It is concluded that the Eskimo viruses were not related to the Lansing prototype and did not stimulate the development of Type 2 antibody in monkeys.

Types 1 and 3 Viruses

Because of the large number of monkeys needed, it was only possible to test two of the pooled antisera (E3-56 and E3-227) against the Brunhilde and Leon prototypes. It will be seen from Table V that these Eskimo antisera completely neutralized 100 PD_{50} of Brunhilde virus, whereas they exerted no neutralizing action on Leon virus.

Conclusion

From the results of experiments carried out by inoculating monkeys or mice with mixtures of the prototype strains for Types 1, 2, and 3 viruses and Eskimo antisera, it is concluded that Strains E3-56 and E3-227 are Type 1 poliomyelitis viruses.

TABLE IV

ANTISERA TO ESKIMO VIRUSES: NEUTRALIZATION TESTS WITH LANSING VIRUS IN MICE

Immunizing strain	Monkeys immunized	Mortalities in groups of mice inoculated with virus and undiluted monkey immune serum obtained at following weeks of immunization program*		
		Week 4	Week 8	Week 12
E3-56	1003	9/9	9/10	9/10
	1004	10/10	10/10	10/10
	1005	10/10	8/10	9/9
E3-79	1007	10/10	10/10	10/10
	1008	10/10	10/10	10/10
	1009	8/10	10/10	10/10
E3-227	1010	10/10	10/10	9/10
	1012	10/10	10/10	10/10
	1013	8/10	9/10	9/10

* *Each virus–serum mixture contained a final amount of 100 LD_{50} of Lansing virus.*

TABLE V

ANTISERA TO ESKIMO VIRUSES: NEUTRALIZATION TESTS WITH BRUNHILDE AND LEON VIRUSES IN MONKEYS

Antiserum to Eskimo strain	Paralysis in monkeys inoculated with mixture of undiluted pooled immune serum to Eskimo strain and 100 PD_{50} of following viruses*	
	Brunhilde	Leon
E3-56	0/5	5/5
E3-227	0/5	5/5

* *Final amount of virus in virus–serum mixture, 100 PD_{50}.*

Part II : Typing in Tissue Culture

MATERIALS AND METHODS

Two methods have been used for the typing of Eskimo strains. In the direct method (Method A) antiserum to each of the prototype viruses was mixed with the virus to be typed, and each virus–serum mixture was then inoculated into a group of roller-tube cultures; inhibition of the virus cytopathogenic effect by one of the prototype antisera indicated the type of the Eskimo strain. In the indirect method (Method B), antiserum prepared in monkeys to the strain being typed was mixed with a strain of each type which exerts a well-marked cytopathogenic effect. Inhibition by the serum of the cytopathogenic changes induced by one of the type strains indicated the type of the strain under investigation.

Method A

Preparation, Maintenance, and Infection of Cultures

Roller-tube cultures were prepared by adding to rimless test tubes (150 mm. × 15 mm.) one or two drops of chick plasma. This fluid was then distributed evenly over the lower third of the tube, and six to eight fragments of monkey testis were embedded therein. One drop of 20% chick embryo extract, as commonly employed in tissue culture techniques, was then added and the tube slowly rotated horizontally until clotting of the plasma was observed.

A quantity of 2 ml. of synthetic mixture No. 199 devised by Morgan, Morton, and Parker (9) and previously employed by us in Maitland-type flask cultures (6, 22) was then added. The tubes were tightly stoppered and placed horizontally in the drum of a mechanical rotating machine, and incubated at 37° C. After seven days, the fragments of testis were seen on microscopic examination to be surrounded by an abundant outgrowth of fibroblasts. The nutrient mixture was then completely removed and replaced with 1.8 ml. of nutrient mixture to which was added 0.2 ml. of virus suspension. The cultures were examined daily for the appearance of cytopathogenic changes in the fibroblasts. With the strains used in this study this effect was usually well-marked by about seven days after addition of virus. Depending on the nature of the experiment, one or more replacements of culture fluid with fresh mixture No. 199 were made at intervals of four to seven days.

Adaptation of Eskimo Strains to Tissue Culture

In order to adapt the three Eskimo strains to growth in tissue culture, roller-tube cultures of monkey testis prepared as just described were infected with 0.2 ml. of a 1-in-10 dilution of the spinal cord pools used in Part I of the typing program (Table I). All three strains produced a cytopathogenic effect within 7 to 14 days. After a period of 16-24 days, during which time the original 2 ml. of nutrient was replaced on four to six occasions, a further series of cultures of monkey testis was infected in order to continue subcultivation in tissue culture. In these first subcultures, the cytopathogenic effect was noted somewhat earlier, and a second series of subcultures was inoculated with fluid removed at the third or fourth fluid change. The fluids removed during this second series of subcultures were pooled, distributed in small vials, and stored in the frozen state. These pools constituted the stocks of the three Eskimo strains used for typing in tissue culture.

Titration of Eskimo Strains

In order to titrate the Eskimo strains adapted as just described, serial 10-fold dilutions of the tissue culture pools were prepared in saline covering the range $10^{-1.0}$ to $10^{-4.0}$. A quantity of 0.2 ml. of each dilution was mixed with 1.8 ml. of mixture No. 199 and inoculated into three roller-tube cultures

showing outgrowths of fibroblasts. These cultures were incubated at 37° C. for seven days, during which time the fluid was not replaced. The tests were then read, with the results shown in Table VI. Titers of virus are expressed in terms of the cytopathogenic dose or CPD, which is defined as the highest dilution of virus causing cytopathogenic changes in all of the three cultures inoculated. It may be here mentioned that the representative strains, Brunhilde (Type 1), Y-SK (Type 2), and Saukett (Type 3) employed in the titration of the prototype antisera and in Method B have been titrated in a similar manner.

TABLE VI

TYPING OF ESKIMO VIRUSES BY METHOD A: TITERS OF VIRUSES AND ANTISERA DETERMINED IN TISSUE CULTURE

Designation of material	CPD titer of viruses*	CPID titer of sera*
Eskimo virus E3-56 (tissue culture)	$10^{-2.0}$	—
Eskimo virus E3-79 (tissue culture)	$10^{-3.0}$	—
Eskimo virus E3-227 (tissue culture)	$10^{-3.0}$	—
Brunhilde antiserum (monkey)	—	$10^{-3.0}$
Lansing antiserum (monkey)	—	$10^{-3.0}$
Leon antiserum (monkey)	—	$10^{-3.0}$

* *Determined in groups of three roller-tube cultures of monkey testis fibroblasts.*

Titration of Prototype Antisera

During the course of other studies in this laboratory, we have prepared pools of serum from rhesus monkeys immunized by repeated intramuscular injections of the Brunhilde, Lansing, and Leon strains in the form of infected monkey spinal cord (15, 23). These sera were titrated in tissue culture in the following manner: Serial 10-fold dilutions of each antiserum were prepared in saline covering the range $10^{-1.0}$ to $10^{-5.0}$. Each dilution was then mixed with an equal volume of a pool of one of the strains referred to above, Brunhilde, Y-SK, or Saukett; the final amount of virus in each 0.2 ml. of virus–serum mixture was 100 CPD. The virus–serum mixtures were allowed to stand at room temperature for one and one-half hours and 0.2 ml. of each mixture and 1.8 ml. of mixture No. 199 were added to each of three roller-tube cultures showing outgrowths of fibroblasts. These cultures were incubated at 37° C. for seven days, during which time the fluids were not replaced. With each serum test, a titration of the virus was also carried out, to check the results of previous titrations. The results were read seven days after infection of the cultures. Titers are shown in Table VI. Titers of serum are expressed in terms of the cytopathogenic inhibiting dose or CPID, which is defined as the highest dilution of serum completely inhibiting cytopathogenic

changes in all of the three cultures inoculated. This titer is expressed, not in terms of the final dilution of serum, but of the serum dilution before admixture with an equal volume of virus suspension. For example, the final dilution of a serum, the CPID titer of which is expressed as $10^{-3.0}$, is actually $10^{-3.3}$ (1 : 2000). This is in conformity with the practice that has been followed for many years in expressing titers of poliomyelitis antisera.

Details of Typing Test

A volume of undiluted Eskimo virus tissue culture pool was mixed with an equal volume of each prototype antiserum diluted 1 : 2, and the virus–serum mixtures were allowed to stand at room temperature for one and one-half hours. As controls, a volume of undiluted Eskimo virus pool was mixed with an equal volume of normal monkey serum (diluted 1 : 2). A quantity of 0.2 ml. of virus–serum mixture, together with 1.8 ml. of mixture No. 199, was then inoculated into groups of three roller-tube cultures (12 tubes in all). It was not necessary to change the nutrient fluid after infection, as cytopathogenic changes were usually evident in seven days.

Method B

Preparation, Maintenance, and Infection of Cultures

Roller-tube cultures were prepared, maintained, and infected exactly as described for Method A above.

Growth of Prototype Viruses in Tissue Culture

The Brunhilde strain was adapted to tissue culture by inoculation of infected monkey spinal cord (23). A pool of infected tissue culture fluids harvested during the second subculture was prepared and stored in the frozen

TABLE VII

TYPING OF ESKIMO VIRUSES BY METHOD B: TITERS OF VIRUSES AND ANTISERA DETERMINED IN TISSUE CULTURE

Designation of material	CPD titer of viruses*	CPID titer of sera*
Brunhilde virus (tissue culture)	$10^{-4.0}$	—
Y-SK virus (tissue culture)	$10^{-4.0}$	—
Saukett virus (tissue culture)	$10^{-4.0}$	—
Eskimo E3-56 antiserum (monkey)	—	$10^{-2.0}$
Eskimo E3-79 antiserum (monkey)	—	$10^{-1.0}$
Eskimo E3-227 antiserum (monkey)	—	$10^{-2.0}$

* *Determined in groups of three roller-tube cultures of monkey testis fibroblasts.*

state. The Y-SK strain was adapted to tissue culture in a similar way by inoculation of infected monkey cord. A pool of infected culture fluids was prepared from second subculture in roller-tube cultures. The Saukett strain was received in the form of infected tissue culture fluid from Dr. Jonas E. Salk, Pittsburgh; a pool was prepared from the second subculture of this strain in our laboratory.

Titration of Prototype Strains

The pools of prototype viruses just described were titrated by the inoculation of serial 10-fold dilutions covering the range $10^{-1.0}$ to $10^{-5.0}$ into groups of three roller-tube cultures. The quantities used were as described for Method A. Titers are expressed in the form of CPD values, and are presented in Table VII.

Titration of Eskimo Antisera

Pools of antisera were prepared to the three Eskimo strains by the inoculation of monkeys as already described in Part I above. Serial 10-fold dilutions of these serum pools were mixed with equal volumes of the homologous Eskimo tissue culture virus pools, so that 0.2 ml. of the mixture contained 100 CPD of virus. The mixtures stood at room temperature for one and one-half hours, and 0.2 ml. of each mixture with 1.8 ml. of mixture No. 199 were then added to each of three roller-tube cultures. CPID titers were determined after seven days' incubation as before. The titer of the antiserum prepared against one of the Eskimo viruses (E3-79) was low ($10^{-1.0}$), and this serum was not used further. The other two sera were more satisfactory, having titers of $10^{-2.0}$ (Table VII).

Details of Typing Test

The objective of this test was to determine whether Eskimo antisera E3-56 and E3-227 would inhibit the cytopathogenic changes induced in tissue cultures by representatives of the three types (Brunhilde, Y-SK, and Saukett). Details of preparation of the antisera, and the representative strains of the three types have already been given. The three Eskimo viruses were also included in the test to determine whether they varied one from another antigenically. The test was set up as follows: Eskimo antisera E3-56 and E3-227 were diluted $10^{-1.0}$, $10^{-2.0}$, and $10^{-3.0}$, and each dilution was mixed with an equal volume of the six virus strains (in the form of pools of infected tissue culture fluids) so that 0.2 ml. of each virus–serum mixture contained 100 CPD of virus. The mixtures were left at room temperature for one and one-half hours; 0.2 ml. of each virus–serum mixture and 1.8 ml. of mixture No. 199 were then inoculated in groups of two roller-tube cultures. Cultures were then incubated at 37° C. as before, and microscopic examination for cytopathogenic changes was carried out seven days later.

Results

Method A

Two of the three strains were typed by this method (E3-79 and E3-227). From Table VIII it will be seen that the cytopathogenic changes induced by these viruses were not inhibited by normal monkey serum or by Lansing or Leon antiserum. The cytopathogenic effect was completely inhibited by Brunhilde (Type 1) antiserum. It is therefore concluded that strains E3-79 and E3-227 both belong to Type 1.

TABLE VIII

TYPING OF ESKIMO VIRUSES BY METHOD A: NEUTRALIZATION OF ESKIMO VIRUSES BY TYPE SPECIFIC ANTISERA

Eskimo strain	Presence of cytopathogenic changes in roller-tube cultures inoculated with mixtures of Eskimo virus and the following sera*†												Type of Eskimo virus
	Normal monkey serum			Type 1 (Brunhilde) antiserum			Type 2 (Lansing) antiserum			Type 3 (Leon) antiserum			
E3-79	+	+	+	−	−	−	+	+	+	+	+	+	1
E3-227	+	+	+	−	−	−	+	+	+	+	+	+	1

* *Sera used in an initial dilution of 1 : 2.*
† *+ sign indicates presence of cytopathogenic changes;*
− sign indicates absence of cytopathogenic changes.
Results are given for each of three cultures separately.

TABLE IX

TYPING OF ESKIMO STRAINS BY METHOD B: NEUTRALIZATION OF TYPE AND ESKIMO STRAINS BY ESKIMO ANTISERA

Strain of virus	Presence of cytopathogenic changes in roller-tube cultures induced by viruses mixed with dilutions of following sera*†											
	Serum E3-56						Serum E3-227					
	$10^{-1.0}$		$10^{-2.0}$		$10^{-3.0}$		$10^{-1.0}$		$10^{-2.0}$		$10^{-3.0}$	
Brunhilde	−	−	−	−	+	+	−	−	−	−	+	+
Y-SK	+	+	+	+	+	+	+	+	+	+	+	+
Saukett	+	+	+	+	+	+	+	+	+	+	+	+
E3-56	−	−	−	−	+	+	−	−	−	−	+	+
E3-79	−	−	+	+	+	+	−	−	+	+	+	+
E3-227	−	−	+	+	+	+	−	−	−	+	+	+

* *A final amount of 100 CPD of virus present in 0.2 ml. of each virus–serum mixture.*
† *+ sign indicates presence of cytopathogenic changes;*
− sign indicates absence of cytopathogenic changes.
Results are given for each of two tubes.

Method B

The results of typing by this method are presented in Table IX, from which it will be seen that the two Eskimo sera inhibited the cytopathogenic effect produced by the Brunhilde strain; because these sera were of low titer, inhibition did not occur with serum dilutions of $10^{-3.0}$, but was noted with serum dilutions of $10^{-1.0}$ and $10^{-2.0}$. The two sera did not inhibit at all the cytopathogenic changes induced by the Y-SK or Saukett strains. As far as can be determined by this type of test, the three Eskimo strains do not differ one from another antigenically, and all belong to Type 1.

Discussion

The outbreak of poliomyelitis that occurred amongst Eskimos living at the small, far northerly settlement of Chesterfield Inlet, N.W.T., in February 1949 was so unusual in respect of the high rate of attack and the epidemiological features, that it seemed to be of value to analyze the antigenic structure of several strains of virus isolated from patients. Five strains have now been typed in the laboratories of Dr. David Bodian and Dr. Jonas Salk, as well as in the Connaught Medical Research Laboratories. As shown in Table X, all strains have been found to belong to Type 1. The five strains typed were isolated from brain and cord; stool; or throat washings. From the results of the typing tests, it may reasonably be concluded that this outbreak was in fact caused by a single antigenic type. The type of virus isolated appears to be the one responsible for the majority of epidemics of poliomyelitis on the North American continent (5). It will be recalled that the Chesterfield Inlet outbreak was probably caused by the introduction of infection by a healthy person who had been in close contact with Eskimos affected with poliomyelitis at a station south of Chesterfield Inlet (1, 13). It seemed likely that infection had been carried to this more southerly Eskimo settlement by Eskimos who had direct or indirect contact with the busy port of Churchill, Man., where poliomyelitis is known to have occurred in the summer of 1948. It would appear that the peculiar characteristics of the Chesterfield Inlet outbreak cannot be attributed to the emergence of an unusual antigenic type of virus, but rather to the operation of some factor which affected the susceptibility of the Eskimos to invasion by poliomyelitis virus. In this connection Sabin (19) suggests that the high paralytic attack rate may have been a reflection of the genetic constitution of the inbred population.

The typing of the Eskimo strains of virus presented a certain amount of difficulty because they were of a low degree of pathogenicity to monkeys by the cerebral route. This difficulty was partly overcome by the method suggested by Salk (20), in which monkeys were immunized with preparations of infected monkey central nervous system incorporated with Freund's adjuvants, and the serum was tested for capacity to neutralize the three Type viruses. This method did not prove entirely satisfactory because, although the monkeys received six injections of virus at two-weekly intervals, only low levels of neutralizing antibody developed.

TABLE X

RESULTS OF TYPING CHESTERFIELD INLET ESKIMO POLIOMYELITIS STRAINS BY FOUR METHODS

Designation of strain	Human pathological specimen used to inoculate monkeys	Results of typing strain by following method				Reference
		In monkeys		In tissue cultures of fibroblasts		
		Type-specific antiserum mixed with Eskimo viruses, and inoculated cerebrally	Eskimo antiserum prepared in monkeys, mixed with type viruses, and inoculated cerebrally	Type-specific antiserum mixed with Eskimo viruses, and inoculated	Eskimo antiserum prepared in monkeys, mixed with type viruses, and inoculated	
E3-56	Brain and cord	—	Type 1	—	Type 1	This article
E3-79	Stool	—	—	Type 1	—	This article
E3-177	Stool	—	Type 1	—	—	(7, 21)
E3-213	Stool	Type 1	—	—	—	(2)
E3-227	Throat washings	—	Type 1	Type 1	Type 1	This article

In the typing of the Chesterfield Inlet strains of virus, the technique of tissue culture proved particularly suitable.

This study emphasizes the extreme value of the tissue culture technique in the typing of poliomyelitis strains. The particular advantages of this technique are the speed and low cost. Furthermore, it can be carried out in laboratories where facilities for the care of monkeys are limited.

Acknowledgments

We wish to express our thanks to Doctors Jonas E. Salk, Pittsburgh, and Joseph L. Melnick, New Haven, who kindly sent us certain strains of poliomyelitis virus.

References

1. Adamson, J. D., Moody, J. P., Peart, A. F. W., Smillie, R. A., Wilt, J. C., and Wood, W. J. Can. Med. Assoc. J. 61 : 339. 1949.
2. Bodian, D. Am. J. Hyg. 54 : 174. 1951.
3. Clark, E. M. and Rhodes, A. J. Can. J. Med. Sci. 29 : 216. 1951.
4. Clark, E. M. and Rhodes, A. J. Can. J. Med. Sci. 30 : 390. 1952.
5. Committee on Typing of the National Foundation for Infantile Paralysis. Am. J. Hyg. 54 : 191. 1951.
6. Franklin, A. E., Duncan, D., Wood, W., and Rhodes, A. J. Proc. Soc. Exptl. Biol. Med. 79 : 715. 1952.
7. Kessel, J. F., Pait, C. F., and Kokko, V. P. Am. J. Hyg. 54 : 205. 1951.
8. Ledinko, N., Riordan, J. T., and Melnick, J. L. Am. J. Hyg. 55 : 323. 1952.
9. Morgan, J. F., Morton, H. J., and Parker, R. C. Proc. Soc. Exptl. Biol. Med. 73 : 1. 1950.
10. Paul, J. R. and Riordan, J. T. Am. J. Hyg. 52 : 202. 1950.
11. Paul, J. R., Riordan, J. T., and Kraft, L. M. J. Immunol. 66 : 695. 1951.
12. Paul, J. R., Riordan, J. T., and Melnick, J. L. Am. J. Hyg. 54 : 275. 1951.
13. Peart, A. F. W. Can. J. Pub. Health, 40 : 405. 1949.
14. Rhodes, A. J., Clark, E. M., Goodfellow, A., and Donohue, W. L. Can. J. Pub. Health, 40 : 418. 1949.
15. Rhodes, A. J., Clark, E. M., Shimada, F. T., Donohue, W. L., and Ritchie, R. C. Can. J. Med. Sci. 30 : 54. 1952.
16. Riordan, J. T., Ledinko, N., and Melnick, J. L. Am. J. Hyg. 55 : 339. 1952.
17. Robbins, F. C., Enders, J. F., and Weller, T. H. Proc. Soc. Exptl. Biol. Med. 75 : 370. 1950.
18. Robbins, F. C., Enders, J. F., Weller, T. H., and Florentino, G. L. Am. J. Hyg. 54 : 286. 1951.
19. Sabin, A. B. Am. J. Pub. Health, 41 : 1215. 1951.
20. Salk, J. E., Lewis, L. J., Youngner, J. S., and Bennett, B. L. Am. J. Hyg. 54 : 157. 1951.
21. Salk, J. E., Youngner, J. S., Lewis, L. J., and Bennett, B. L. Am. J. Hyg. 54 : 255. 1951.
22. Thicke, J. C., Duncan, D., Wood, W., Franklin, A. E., and Rhodes, A. J. Can. J. Med. Sci. 30 : 231. 1952.
23. Wood, W., Franklin, A. E., Clark, E. M., and Rhodes, A. J. Proc. Soc. Exptl. Biol. Med. 81 : 434. 1952.
24. Youngner, J. S., Lewis, L. J., Ward, E. N., and Salk, J. E. Am. J. Hyg. 55 : 347. 1952.
25. Youngner, J. S., Ward, E. N., and Salk, J. E. Am. J. Hyg. 55 : 291. 1952.
26. Youngner, J. S., Ward, E. N., and Salk, J. E. Am. J. Hyg. 55 : 301. 1952.

This symposium was sponsored by the Biochemistry Subject Division of the Chemical Institute of Canada in celebration of the centenary of the incorporation of Laval University of Quebec City. The symposium was held at Laval University on October 9th, 1952, with Dr. Orville F. Denstedt of McGill University as chairman.

CHEMISTRY OF THE NUCLEIC ACIDS[1]

By Gordon Butler

Two types of nucleic acid are found in nature: the ribo- or pentose type and the desoxyribo- or desoxy-pentose type. I will discuss each type separately.

Ribonucleic Acid

Complete hydrolysis of ribonucleates yields a mixture of four nitrogenous bases, the two pyrimidines, cytosine and uracil, and the two purines, adenine and guanine. Numerous careful investigations in recent years have failed to reveal the presence of other bases and we may conclude that only these four are present. Another product of hydrolysis of the ribonucleates is D(−)ribose, first identified by Levene and Jacobs (47) and later, with more certainty, by Barker and Gulland (4) in yeast ribonucleate. The same sugar has also been found in the ribonucleates from liver (20), pancreas, and tubercle bacillus (72). One more product of complete hydrolysis is inorganic phosphate.

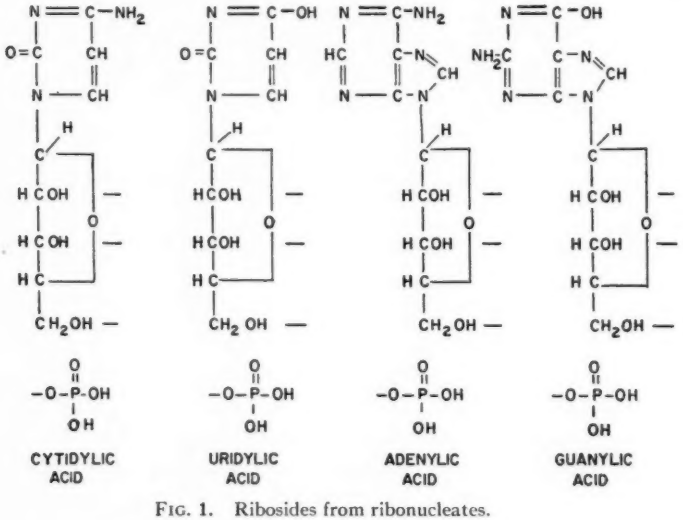


FIG. 1. Ribosides from ribonucleates.

[1] *Manuscript received December 16, 1952.*
Contribution from the Department of Biochemistry, University of Toronto, Toronto, Ontario.

I am going to consider the nucleotide as the unit structural element in the nucleic acid. Reference to Fig. 1 will illustrate how the various components of the nucleotides are arranged in the molecule. Partial hydrolysis of ribonucleate may yield nucleosides (base + sugar) or nucleotides (nucleoside phosphorylated on the sugar). Let us first consider the details of the nucleoside structure. Gulland obtained spectroscopic evidence (29) and Todd and his colleagues proved by synthesis (35, 34, 55) that the sugar was attached at position 3 of the pyrimidine ring and at position 9 of the purine ring. The synthetic work of Todd and his colleages indicated that the glycosidic link is the β type (21); these workers also showed by periodate oxidation (34) and by synthesis (42, 22) that the ribose is present in the furanose form.

Present ideas about the point of union of the phosphate in the nucelotide are much less clear-cut. It can be seen from Fig. 1 that there are three possibilities; it may be at carbon 2′, 3′, or 5′ of the ribose and all three possible compounds have been isolated. As a result of the work of Levene (46) it has been customary for many years to consider the ribomononucleotides as 3′-phosphates but this view is no longer tenable. Cohn and his colleagues (15) have shown that in an alkaline hydrolyzate of ribonucleic acid each of the four mononucleotides occurs as a mixture of two isomers which Cohn has designated *a* and *b*. The difference between these pairs of isomers is due to the attachment of the phosphate at position 2′ and 3′ of the sugar. It has not yet been possible to determine the positions of the phosphates in these two isomers (8) because an unequivocal synthesis of nuceloside-2′-phosphate or -3′-phosphate has not yet been achieved. Therefore in this important

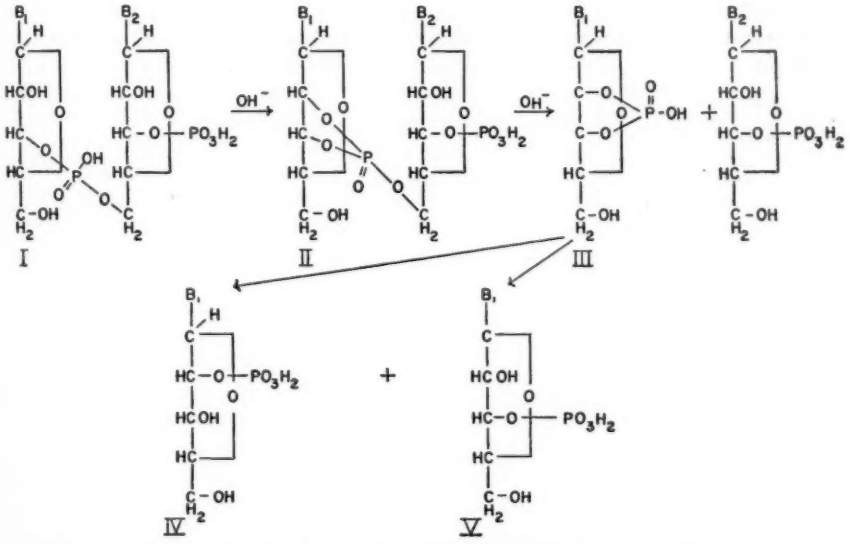


FIG. 2. Alkaline hydrolysis of ribose polynucleotide through a cyclic phosphate ester.

detail we are still ignorant of the structure of ribonucleates. A little later I will outline a theory that has been advanced to explain the occurrence of these two isomers. The 5′-phosphates of the ribose nucleosides have been obtained by Cohn and Volkin by the hydrolysis of ribonucleate with a purified diesterase from snake venom (18). It is possible to establish the position of the phosphate in this case by using specific 5′-nucleotidases present in snake venom (38) and bull testis (32).

These findings immediately give a lead as to the nature of the internucleotide linkages in the macromolecule. Since one method of hydrolysis yields nucleotides that are 2′- (or 3′-) phosphates and another yields 5′-phosphates, adjacent nucleosides must be linked through a phosphate diester bridge attached at one end to the 5′-carbon of the ribose and at the other end to the 2′- (or 3′-) carbon. Brown and Todd (8) have advanced the following theory to explain the production of a mixture of 2′- and 3′-phosphates on alkaline hydrolysis of such a phosphate diester.

The first action of alkali on a hypothetical dinucleotide such as that illustrated (I) is the formation of the triply esterified cyclic phosphate (II). This causes the P-O-C_5' linkage to be unstable and to undergo hydrolysis leaving the cyclic phosphate III. The latter then undergoes further breakdown, the P-O-C_2' and the P-O-C_3' linkages being hydrolyzed randomly to yield a mixture of the two nucleotides, IV and V, in approximately equal amounts. This theory has been warmly received because it clarifies a number of problems encountered in the hydrolysis of ribonucleates. Furthermore, Markham and Smith (57) have isolated from ribonuclease digests of yeast ribonucleate, two substances with the properties expected for a hypothetical intermediate similar to III.

Now let us consider some details of structure of the whole ribonucleate molecule. A convenient starting place for the discussion is the formula

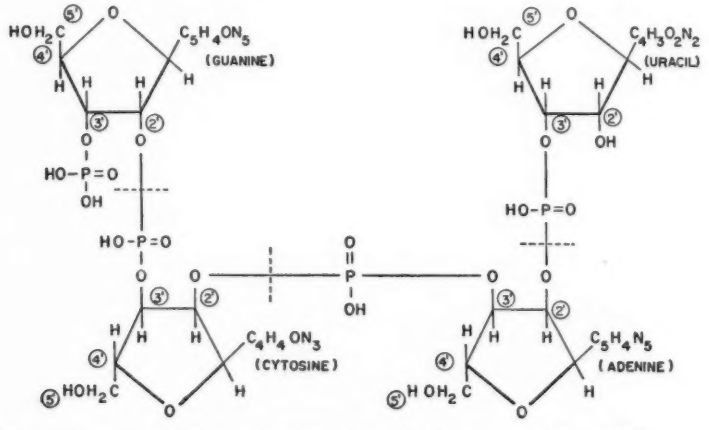


FIG. 3. Structure of ribonucleic acid, proposed by Levene and Tipson (52).

proposed by Levene and Tipson in 1935 (52) for yeast ribonucleic acid; this represents the point of departure of the new advances in our knowledge. It is immediately obvious that this formula showing the internucleoside link between C_2' and C_3' is incorrect.

Several different kinds of experimental evidence have been brought to bear on the problem of the structure of ribonucleate. I should like to consider each of these and its implications.

Molecular Weight Determinations

Most of the early methods of preparing nucleates made use of heat and strong acids and alkalies (see (45)) which led to degradation of the molecule and a decrease in molecular weight. A few of the more recent estimations of molecular weight are given in Table I. It can readily be seen that there is no reason to consider either of the ribonucleates listed as a tetranucleotide.

TABLE I

MOLECULAR WEIGHTS OF RIBONUCLEATES

Material	Molecular weight	Nucleotides per molecule	Reference
Yeast RNA	17,000	58–88	54
Tobacco mosaic virus RNA	37,000	116	
Yeast RNA	10,000	30	25
Tobacco mosaic virus RNA			
Fresh	300,000	900	
After spontaneous decomposition	61,000	180	14
After treatment with cold alkali	15,000	45	
Yeast RNA from various sources	10,000–23,000	28–80	26

Analysis for Bases

During the past four years several samples of ribonucleate have been carefully hydrolyzed and the bases so liberated have been separated chromatographically and estimated by spectrophotometry. The results of these analyses are presented in Table II.

In presenting all of the analytical results to be found in the literature it was necessary to make certain arithmetical manipulations of some of the data so that they would conform to the method of presentation in Table II. In such cases the analytical values listed may be somewhat too high since no account was taken of the losses incurred during analysis.

Very few generalizations can be made about these results. It can be seen that there are variations in composition in different tissues of the same species, in the same tissue of different species, and even in the same tissue of the same species when the ribonucleate was prepared and analyzed in different laboratories. There are almost certain to be errors in some of these estimations

TABLE II

COMPOSITION OF RIBONUCLEATES

Source		Moles per 100 moles nucleotide				Reference
		Cytosine		Adenine	Uracil	Guanine	
Species	"Organ"	5-Methyl- Cyt.					
Ox	Spleen	31.7		17.9	15.4	35.3	74
	Thymus	25.7		18.4	12.1	44.0	74
	Pancreas	25.2		20.1	5.6	49.2	72
	Liver	29.5		19.1	16.3	35.1	74
Rabbit	Liver	29.4		18.0	17.4	35.0	74
Rat	Liver	33.0		17.9	25.8	33.2	74
Mouse	Liver	26.9		20.9	18.0	34.0	74
Chicken	Rous sarcoma	28.5		14.2	9.0	48.2	5
	Reynals "D" sarc.	29.8		12.7	10.8	45.8	5
Yeast		19.9		28.0	20.5	31.6	61
		0	29.1	31.0	9.7	30.1	78, 72
		20.0		25.8	24.2	31.2	67
Viruses							
Aucuba mosaic		19.5		30.0	26.2	23.7	56
Tobacco mosaic		15.5		31.0	24.0	29.2	56
Rib grass		17.2		29.2	26.2	27.0	56
Cucumber 4		18.5		26.0	29.7	25.7	56
Tomato mosaic		20.0		27.0	25.7	27.0	56, 58
Turnip yellow mosaic		38.2		22.7	22.2	17.2	58

because of the difficulties of the analytical procedure. Nevertheless, it is apparent that the bases are not present in regular or simple proportions and that the structures of the ribonucleates must be much more complex than that suggested by Levene (Fig. 3).

Electrometric Titrations

Levene was led to the formulation of his tetranucleotide structure mainly as a result of his studies of electrometric titration (50). In these he found that yeast ribonucleic acid has one secondary and four primary phosphoryl dissociations for every four atoms of phosphorus, as shown in the structure presented in Fig. 3. It must be remembered, however, that this applies only to material with a molecular weight of about 1400. More recently, Gulland and his colleagues (27) found on titrating yeast ribonucleate that for every four atoms of phosphorus, there were three primary and one secondary phosphoryl dissociations, i.e., about 25% of the titratable phosphate was secondary. Zittle (83) in similar studies obtained a value of 18%. Such a high proportion of end groups (secondary phosphoryls) in a substance of high molecular weight indicates a branched chain structure in the macromolecule. The fact that there is a corresponding deficiency of primary phosphate dissociations indicates that the branching may take place through triply esterified phosphoric acid.

Hydrolysis with Phosphomonoesterase

The phosphate end groups on the branch chains may be removed by treating the intact ribonucleate with a phosphomonoesterase which acts specifically on singly esterified phosphate groups. A quantitative estimate of the amount of branching may then be obtained by measuring the proportion of the phosphate liberated in the inorganic form. Experiments of this kind have been carried out on yeast ribonucleate by Gulland and Jackson (30) using bone phosphatase, and by Schmidt *et al.* (64) with prostatic acid phosphatase. Cohn, Doherty, and Volkin treated calf liver ribonucleate with bone phosphatase (17). In the three studies the amounts of inorganic phosphate liberated were found to be, respectively, 6.5%, 8%, and 10%.

Methylation

Another type of branching in the ribonucleate molecule has been indicated by the methylation studies of Anderson, Barker, Gulland, and Lock (2). They obtained from the hydrolyzate of methylated ribonucleate, equal amounts of ribose, monomethyl ribose, and dimethyl ribose. The most obvious explanation of this result is that two types of branching exist. The ribose residues along the main chain of the molecule, and not concerned with branching, acquire only one methyl group. The ribose units at the ends of branches formed by triple esterification of the phosphate would contain two methylated positions. To explain the presence of free ribose it must be assumed that there is a second type of branching which arises from the triple phosphorylation of ribose.

Other Enzymic Degradations

The degradation of ribonucleate with purified snake venom diesterase (18) has already been mentioned. Analysis of the digests showed that 60-70% of the total bases was liberated as the 5′-mononucleotides, while the remaining 30-40% consisted of a mixture of equimolecular amounts of purine nucleosides and pyrimidine nucleoside diphosphates. The last two components may arise from a branched chain of the second type mentioned above; the pyrimidine nucleoside diphosphates could arise from the points of branching.

Further knowledge of ribonucleate structure has been provided by examination of the products of ribonuclease digestion (57, 59, 64, 75, 17). In such hydrolyzates 60% of the total pyrimidines appeared as mononucleotides while the remainder of the pyrimidines and all of the purines were present in the larger fragments – the oligonucleotides. All of these so far examined (di, tri, and tetra-nucleotides) have contained the pyrimidine nucleotide as the end group bearing the singly esterified phosphate. The other bases in the oligonucleotides were purines. Thus ribonuclease is a specific pyrimidine nucleotide diesterase.

Two other findings of interest have come out of these studies. First, all the end-groups and the free pyrimidine nucleotides are of the *b* type (17) and therefore the internucleotide linkage in the straight chain of the ribonucleate

must be of the 5′-P-*b* type. Secondly, the ribonuclease acts through the intermediate formation of a cyclic anhydride as illustrated in Fig. 2 (57, 6).

All of these findings about the structure of ribonucleate and its cleavage by various reagents may be summarized by reference to a hypothetical structure presented by Cohn, Doherty, and Volkin (17) by way of illustration.

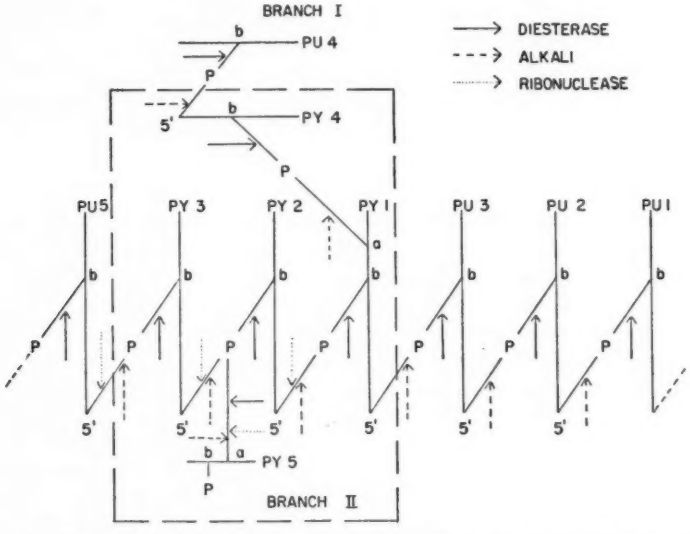


FIG. 4. Hypothetical diagram of ribonucleic acid structure proposed by Cohn, Doherty, and Volkin (17).

This diagram may be used to illustrate some of the findings already mentioned:

1. On methylation the ribose of PY-1 would be unchanged; that of PU-4, doubly methylated, and elsewhere, singly methylated.
2. Snake venom diesterase would liberate all of the nucleotides as the 5′-phosphates with the exception of PU-4 (as a nucleoside) and PY-1 (as a 5′, *a*-diphosphate).
3. Ribonuclease would liberate PY-2, PY-3, and PY-5 as *b*-mononucleotides (60% of the total) while PY-4 and PY-1 would remain as end-groups on oligonucleotides composed otherwise of purine nucleotides.

Desoxyribonucleic Acids

Until recently desoxyribonucleates were known to contain only four nitrogenous bases – cytosine, thymine, adenine, and guanine. About four years ago, however, Hotchkiss (33) and Wyatt (78, 79) discovered the presence of 5-methyl cytosine in hydrolyzates of desoxyribonucleate. Later, 5-methyl desoxycytidylic acid (16) and 5-methyl desoxycytidine (23) were isolated from

the same source. Levene and his colleagues (49, 48) first identified as 2-desoxy-D-ribose the sugar obtained from desoxyguanosine and this identification was confirmed by Kent (43) for the product of mercaptanolysis of thymus desoxyribonucleate. Chargaff *et al.* (12) were able to demonstrate, by means of paper chromatography, only one sugar (presumably desoxyribose) in the hydrolyzates of thymus and spleen desoxyribonucleate. Phosphate is also present in desoxyribonucleates.

In Fig. 5 are illustrated the structures of the five mononucleotides obtained on hydrolysis of thymus desoxyribonucleate. It might now be of interest to examine these formulae to determine which details of structure are known with certainty. Since convenient methods for the isolation of these mononucleotides (39, 76) and the corresponding nucleosides (63, 62, 3) have become available only recently, they have received relatively little chemical study. Initially it was postulated that the desoxyribose residues were attached to the same nitrogen atoms (N_3 of the pyrimidines and N_9 of the purines) as were the ribose residues in the ribosides from ribonucleate. This theory is supported by the finding that the corresponding ribose- and desoxyribosenucleotides have similar absorption spectra (39, 31), although the suggested structures have not yet been verified by synthesis. For this reason also it cannot be stated whether the glycoside is of the α or β type. Levene and Tipson (51, 52) and Brown and Lythgoe (7) have found that the sugar contains no adjacent hydroxyl groups and therefore has a furanose ring.

It has proved relatively easy to establish the position of the phosphate at C 5′ in the desoxyribosenucleotide since only two positions are available and it was found that the nucleotides are readily dephosphorylated by specific 5′-nucleotidases from snake venom (37) and from bull testis (10).

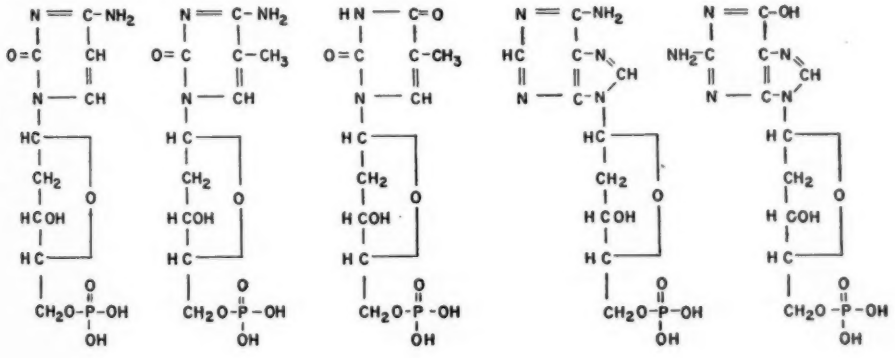


FIG. 5. Mononucleotides obtained from thymus desoxyribonucleate.

Many, if not all, of the internucleotide linkages in the desoxyribonucleate are probably phosphate diesters between C 3′ and C 5′ of adjacent desoxyribose units. The most direct evidence for this is the identification by Levene (44), of the diphosphates of thymidine and desoxycytidine in an acid hydrolyzate of thymus desoxyribonucleate. Little and Butler (53) have, however, suggested that another type of linkage may be present involving the enolic hydroxyls of thymine or guanine, a view criticized by Jordan (41) and Altman and Dounce (1).

The structure of a tetranucleotide unit of desoxyribonucleic acid, as proposed by Levene and Tipson (52), is shown in Fig. 6. It illustrates the 3′-, 5′-phosphate diester links between the nucleosides. It should be noted in this structure that there are no free sugar hydroxyls in the nucleotides in the central part of the molecule. For this reason the formation of a cyclic phosphate is not possible. Brown and Todd (8) have given this as the reason why desoxyribonucleates are not hydrolyzed to mononucleotides by alkali in the same way as are the ribonucleates.

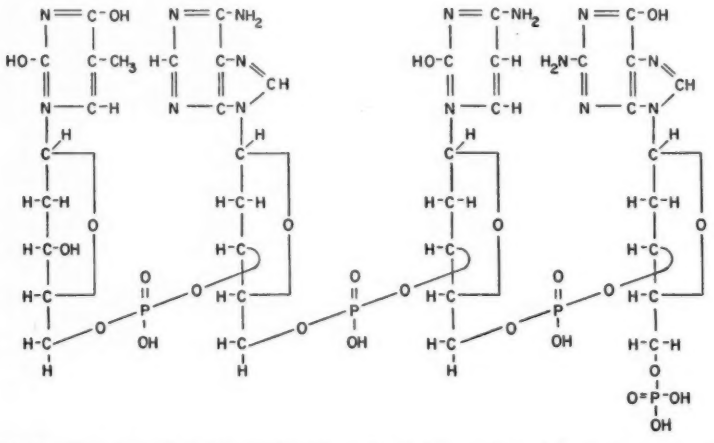


FIG. 6. Structure of a tetranucleotide unit of desoxyribonucleic acid, proposed by Levene and Tipson (52).

Levene's formula, as shown, gives no indication of the size of the desoxyribonucleate molecule. It has been known for many years that the molecular weight of the substance depends on the method of preparation, the more drastic (extremes of pH, high temperature) methods yielding material of smaller particle size. The estimates of the molecular weight (71, 66) range from 1500 to several million.

Electrometric titration of carefully prepared desoxyribonucleate (see (41)) has shown that for each four atoms of phosphorus there are four primary phosphoryl dissociations. Secondary phosphoryl dissociations may also be

present, the number having been variously estimated as one in 10-30 nucleotides. Such a finding indicates the presence in the macromolecule of branches arising in a triply esterified phosphate.

There is now abundant evidence that desoxyribonucleates very rarely contain equimolecular amounts of the four bases as implied in Fig. 6. In the first place, the presence of 5-methyl cytosine in some of the desoxyribonucleates must be taken into consideration. In the second place, recent analyses of desoxyribonucleates show that only rarely do the bases (cytosine, thymine, adenine, and guanine) occur in approximately equimolecular amounts. A summary of the analytical results is presented in Table III. In cases where the desoxyribonucleate has been analyzed for 5-methyl cytosine the results are given; in all other cases (if 5-methyl cytosine is present) the figures given for cytosine represent the total of cytosine and 5-methyl cytosine.

TABLE III

COMPOSITION OF DESOXYRIBONUCLEATES

Source		Moles per 100 moles nucleotide					
		Cytosine		Adenine	Thymine	Guanine	Reference
Species	"Organ"	5-Methyl-	Cyt.				
Man	Spleen		19.7	28.2	28.4	20.3	40
Horse	Spleen		19.2	28.3	26.2	21.9	19
Ox	Spleen		19.0	29.5	27.5	23.8	12
	Thymus		20.0	27.8	27.5	22.9	40, 12, 19
		1.2	21.0	27.7	28.4	21.4	80, 40
	Kidney		20.3	27.4	27.3	21.9	19
	Liver		21.0	26.2	26.9	21.5	40
	Pancreas		21.2	27.2	27.9	21.4	40
	Testis		21.4	25.9	26.7	22.0	40
	Sperm	1.3	20.7	28.7	27.2	22.2	80
Sheep	Spleen		19.6	26.0	26.6	20.7	19
	Sperm	1.0	21.0	28.7	27.2	22.0	80
Rat	Bone marrow	1.1	20.5	28.7	28.5	21.5	80
Chicken	Erythrocytes		20.5	27.1	27.5	20.3	19, 40
Turtle	Erythrocytes		22.2	29.9	29.1	22.9	19
Trout	Sperm		19.9	29.4	27.1	22.2	19
Herring	Sperm	1.9	20.7	27.7	27.5	22.2	80
Salmon	Sperm		20.4	29.7	29.2	20.8	11
Shad	Testis		19.0	26.4	27.2	20.3	19
Sea urchin	Sperm		18.1	26.7	30.8	18.3	19
Locust	(Whole)	0.2	20.7	29.2	29.2	20.5	80
Wheat	Germ	5.5	16.9	26.7	27.5	23.3	80, 40
Yeast			17.5	31.6	33.3	17.5	73
Bacteria							
Pneumo. III			16.7	27.6	29.2	19.0	19
E. coli		0	25.7	22.9	27.3	24.0	70
T.B.		0	33.6	18.0	19.5	28.7	70
Serratia marcescens			32.0	20.6	20.3	27.0	13
Bacillus Shatz			31.7	20.0	19.7	29.5	13
Viruses							
E. coli "phages"		0	11.2	33.5	35.5	20.0	70
Gypsy moth polyhedral virus		0	28.0	21.5	20.2	30.0	70

The figures in Table III illustrate the following points of interest:

1. All the desoxyribonucleates of animal origin are strikingly similar in composition. Although the contents of Table III do not show it, analyses carried out by one procedure and by one investigator reveal small but statistically significant differences in the compositions of desoxyribonucleates from different species. No such differences can be found, however, in the compositions of desoxyribonucleates from different tissues of the same species.
2. Wheat germ desoxyribonucleate appears to be unique in containing a large amount of 5-methyl cytosine.
3. The desoxyribonucleates obtained from yeast and bacteria seem to be different from those of animal origin. The former are all much more variable in composition, some resembling animal desoxyribonucleates in having the adenine and thymine predominant over cytosine and guanine, *Escherichia coli* having approximately equal amounts of the four bases, and others having a preponderance of cytosine and guanine compared to adenine and thymine.
4. In all cases the total of "cytosine" and adenine is equal to the total of thymine and guanine. This means that the number of amino groups in the 6-position is the same as the number of 6-hydroxyl groups in all samples of desoxyribonucleate. It has been pointed out by Butler (9) that this may be important in intermolecular hydrogen bonding which could produce a long, rigid molecule.

It is necessary to make some special mention of the "cytosine" content of the *E. coli* bacteriophages. As recorded in Table III Smith and Wyatt (70) measured the cytosine content of the T_2 and T_6 coliphages. They found that these bacteriophages contained no 5-methyl cytosine. Weed and Cohen (77) reported the isolation of desoxycytidylic acid from these bacteriophages. Marshak (60), however, was unable to find cytosine among the hydrolytic products of these bacteriophages. Recently, Wyatt and Cohen (81) have found that the *E. coli* bacteriophages, T_2 and T_6, contain a previously unknown base, 5-hydroxymethylcytosine, but no cytosine, 5-methylcytosine, nor uracil. This newly found pyrimidine could not be detected in the desoxyribonucleates of thymus or *E. coli*.

There is a possibility that some of the desoxyribonucleates contain still another base, namely uracil. It was encountered as the nucleoside, by Todd and his colleagues, in enzymic hydrolyzates of the desoxyribonucleate from wheat germ (23, 24) and from herring sperm (3). There remains the possibility that this arises by deamination of cytosine during the preparation or hydrolysis of the desoxyribonucleate.

Attempts are now being made in several laboratories to learn how the nucleotides, containing these bases, are arranged in the macromolecule. The approach to this problem has so far been made by examination of the products resulting from the action of desoxyribonuclease on desoxyribonucleates. It was found by the titration of such products (usually called oligonucleotides)

with alkali (25, 53) that they contained on the average four mononucleotides per molecule. Direct evidence of the heterogeneity of these mixtures was provided by the work of Zamenhof and Chargaff (82) who found that such digests contained dialyzable and undialyzable components which differed in the content of nitrogenous bases.

More recent studies, in which mixtures of hydrolytic products have been analyzed by chromatography (65, 36, 69, 68, 28), have shown that they contain a number of di- and tri- (but no mono-) nucleotides. The ones so far identified are: A-C, T-C, G-C, C-C, A-T, G-T, C-T, T-T, G-MC, A-A, A-G, A-C-T, and A-MC-T. (A = desoxyadenylic acid, G = desoxyguanylic, C = desoxycytidylic, MC = 5-methyl desoxyctidylic, and T = thymidylic, the last-designated nucleotide in the compound being the one bearing the singly-esterified phosphate ester.) This work has not yet progressed far enough to shed much light on the arrangement of the nucleotides in the desoxyribonucleates. It does, however, indicate that there is a much more complex pattern than that suggested by Levene (Fig. 6). This type of study should also provide information about the nature and the specificity of the action of desoxyribonuclease. Almost all of the di- and tri-nucleotides, so far identified, have pyrimidine end groups. It might be postulated therefore that the desoxyribonuclease, like ribonuclease, is a pyrimidine-specific phosphodiesterase. Such a postulation would, however, be very difficult to reconcile with the finding of many oligonucleotides containing two adjacent pyrimidine nucleotides (see above).

In concluding this discussion of the chemistry of the nucleic acids it seems advisable to urge caution in applying the results of studies on the identification of the degradation products of nucleates to the formulation of the structure of the whole nucleate molecule. Caution is warranted because there are *a priori* grounds for believing that many samples of ribo- and desoxyribonucleate, especially those from somatic cells, are mixtures. Thus, deductions made, about the position of the different nucleotides, from the identification of oligonucleotides or from the molecular proportions of the bases contained in the nucleate, may give only an over-all picture for the mixture. Such a situation is not so apt to arise in the case of virus nucleates which are more biologically homogeneous, but it may explain the apparent and astonishing uniformity of composition observed in the animal desoxyribonucleates.

References

1. Altman, K. I. and Dounce, A. L. Ann. Rev. Biochem. 21 : 43. 1952.
2. Anderson, A. S., Barker, G. R., Gulland, J. M., and Lock, M. V. J. Chem. Soc. 369. 1952.
3. Anderson, W., Dekker, C. A., and Todd, A. R. J. Chem. Soc. 2721. 1952.
4. Barker, G. R. and Gulland, J. M. J. Chem. Soc. 625. 1943.
5. Beale, R. N., Harris, R. J. C., and Roe, E. M. F. J. Chem. Soc. 1034. 1952.
6. Brown, D. M., Dekker, C. A., and Todd, A. R. J. Chem. Soc. 2715. 1952.
7. Brown, D. M. and Lythgoe, B. J. Chem. Soc. 1990. 1950.
8. Brown, D. M. and Todd, A. R. J. Chem. Soc. 52. 1952.
9. Butler, J. A. V. Endeavour, 11 : 154. 1952.
10. Carter, C. E. J. Am. Chem. Soc. 74 : 1537. 1951.

11. CHARGAFF, E., LIPSHITZ, R., GREEN, C., and HODES, M. E. J. Biol. Chem. 192 : 223. 1951.
12. CHARGAFF, E., VISCHER, E., DONIGER, R., GREEN, C., and MISANI, F. J. Biol. Chem. 177 : 405. 1949.
13. CHARGAFF, E., ZAMENHOF, S., BRAWERMAN, G., and KERIN, L. J. Am. Chem. Soc. 72 : 3825. 1950.
14. COHEN, S. S. and STANLEY, W. M. J. Biol. Chem. 144 : 589. 1942.
15. COHN, W. E. J. Am. Chem. Soc. 72 : 1471, 2811. 1951.
16. COHN, W. E. J. Am. Chem. Soc. 73 : 1539. 1951.
17. COHN, W. E., DOHERTY, D. G., and VOLKIN, E. *In* MCELROY, W. D. and GLASS, B. Symposium on Phosphorus Metabolism, 2 : 352. 1952.
18. COHN, W. E. and VOLKIN, E. Arch. Biochem. and Biophys. 35 : 465. 1952.
19. DALY, M. M., ALFREY, V. G., and MIRSKY, A. E. J. Gen. Physiol. 33 : 497. 1950.
20. DAVIDSON, J. N. and WAYMOUTH, C. Biochem. J. 38 : 375. 1944.
21. DAVOLL, J., LYTHGOE, B., and TODD, A. R. J. Chem. Soc. 833, 967. 1948.
22. DAVOLL, J., LYTHGOE, B., and TODD, A. R. J. Chem. Soc. 969. 1948.
23. DEKKER, C. A. and ELMORE, D. T. J. Chem. Soc. 2864. 1951.
24. DEKKER, C. A. and TODD, A. R. Nature, 166 : 557. 1951.
25. FISCHER, F. G., BÖTTGER, I., and LEHMANN-ECHTERNACHT, H. Z. physiol. Chem. 271 : 246. 1941.
26. FLETCHER, W. E., GULLAND, J. M., JORDAN, D. O., and DIBBEN, H. E. J. Chem. Soc. 30. 1944.
27. FLETCHER, W. E., GULLAND, J. M., and JORDAN, D. O. J. Chem. Soc. 33. 1944.
28. GORDON, A. H. and REICHARD, P. Biochem. J. 48 : 569. 1951.
29. GULLAND, J. M. J. Chem. Soc. 1722. 1938.
30. GULLAND, J. M. and JACKSON, E. M. J. Chem. Soc. 1492. 1938.
31. GULLAND, J. M. and STORY, L. F. J. Chem. Soc. 259, 692. 1938.
32. HEPPEL, L. A. and HILMOE, R. J. J. Biol. Chem. 188 : 665. 1951.
33. HOTCHKISS, R. D. J. Biol. Chem. 175 : 315. 1948.
34. HOWARD, G. A., KENNER, G. W., LYTHGOE, B., and TODD, A. R. J. Chem. Soc. 855, 861. 1946.
35. HOWARD, G. A., LYTHGOE, B., and TODD, A. R. J. Chem. Soc. 556, 1052. 1945.
36. HURST, R. O. Ph.D. Thesis, University of Toronto, 1952.
37. HURST, R. O. and BUTLER, G. C. Federation Proc. 10 : 201. 1951.
38. HURST, R. O. and BUTLER, G. C. J. Biol. Chem. 193 : 91. 1951.
39. HURST, R. O., LITTLE, J. A., and BUTLER, G. C. J. Biol. Chem. 188 : 695. 1951.
40. HURST, R. O., MARKO, A. M., and BUTLER, G. C. J. Biol. Chem. In press.
41. JORDAN, D. O. Ann. Rev. Biochem. 21 : 233-234. 1952.
42. KENNER, G. W., TAYLOR, C. W., and TODD, A. R. J. Chem. Soc. 1620. 1949.
43. KENT, P. W. Nature, 166 : 442. 1950.
44. LEVENE, P. A. J. Biol. Chem. 48 : 119. 1921.
45. LEVENE, P. A. and BASS, L. W. Nucleic acids. The Chemical Catalog Company, Inc., New York. 1931. pp. 294-308.
46. LEVENE, P. A. and HARRIS, I. F. J. Biol. Chem. 101 : 419. 1933.
47. LEVENE, P. A. and JACOBS, W. A. Ber. 42 : 2102, 2469, 2474, 2703. 1909.
48. LEVENE, P. A., MIKESKA, L. A., and MORI, T. J. Biol. Chem. 85 : 785. 1930.
49. LEVENE, P. A. and MORI, T. J. Biol. Chem. 83 : 803. 1929.
50. LEVENE, P. A. and SIMMS, H. S. J. Biol. Chem. 70 : 327. 1926.
51. LEVENE, P. A. and TIPSON, R. S. Z. physiol. Chem. 234 : V. 1935.
52. LEVENE, P. A. and TIPSON, R. S. J. Biol. Chem. 109 : 623. 1935.
53. LITTLE, J. A. and BUTLER, G. C. J. Biol. Chem. 188 : 695. 1951.
54. LORING, H. S. J. Biol. Chem. 130 : 251. 1939.
55. LYTHGOE, B., SMITH, H., and TODD, A. R. J. Chem. Soc. 355. 1947.
56. MARKHAM, R. and SMITH, J. D. Biochem. J. 46 : 513. 1950.
57. MARKHAM, R. and SMITH, J. D. Nature, 168 : 406. 1951.
58. MARKHAM, R. and SMITH, J. D. Biochem. J. 49 : 401. 1951.
59. MARKHAM, R. and SMITH, J. D. Biochem. J. 50 : XXXVIII. 1952.
60. MARSHAK, A. Proc. Natl. Acad. Sci. U.S. 37 : 299. 1951.
61. MARSHAK, A. and VOGEL, H. J. J. Biol. Chem. 189 : 597. 1951.
62. REICHARD, P. and ESTBORN, B. Acta Chem. Scand. 4 : 1047. 1950.
63. SCHINDLER, O. Helv. Chim. Acta, 32 : 979. 1949.
64. SCHMIDT, G., CUBILES, R., ZOLLNER, N., HECHT, L., STRICKLER, N., SERAIDARIA , K., SERAIDARIAN, M., and THANNHAUSER, S. J. J. Biol. Chem. 192 : 715. 1951.
65. SINSHEIMER, R. L. and KOERNER, J. F. J. Am. Chem. Soc. 74 : 283. 1952.
66. SMITH, D. B. and SHEFFER, H. Can. J. Research, B, 28 : 96. 1950.
67. SMITH, J. D. and MARKHAM, R. Biochem. J. 46 : 509. 1950.
68. SMITH, J. D. and MARKHAM, R. Nature, 170 : 120. 1952.
69. SMITH, J. D. and MARKHAM, R. Biochim, et Biophys. Acta, 8 : 350. 1952.

70. SMITH, J. D. and WYATT, G. R. Biochem. J. 49 : 144. 1951.
71. TIPSON, R. S. Advances in Carbohydrate Chem. 1 : 244. 1945.
72. VISCHER, E. and CHARGAFF, E. J. Biol. Chem. 176 : 715. 1948.
73. VISCHER, E., ZAMENHOF, S., and CHARGAFF, E. J. Biol. Chem. 177 : 429. 1949.
74. VOLKIN, E. and CARTER, C. E. J. Am. Chem. Soc. 73 : 1516. 1951.
75. VOLKIN, E. and COHN, W. E. Federation Proc. 11 : 303. 1952.
76. VOLKIN, E., KHYM, J. X., and COHN, W. E. J. Am. Chem. Soc. 73 : 1533. 1951.
77. WEED, L. L. and COHEN, S. S. J. Biol. Chem. 192 : 693. 1951.
78. WYATT, G. R. Nature, 166 : 237. 1950.
79. WYATT, G. R. Biochem. J. 48 : 581. 1951.
80. WYATT, G. R. Biochem. J. 48 : 584. 1951.
81. WYATT, G. R. and COHEN, S. S. Nature, 170 : 1072. 1952.
82. ZAMENHOF, S. and CHARGAFF, E. J. Biol. Chem. 187 : 1. 1950
83. ZITTLE, C. A. J. Biol. Chem. 166 : 491. 1946.

DISCUSSION

Question—**Dr. D. Beall** *(Ayerst, McKenna and Harrison Ltd., Montreal)*: Is there a possibility that some of the isolated degradation products of the nucleic acids may be artefacts?

Answer—**Dr. Butler** (*University of Toronto*): From time to time artefacts have been found among the products of hydrolysis of nucleic acids. The most recent and careful studies have, however, revealed the presence of only the substances as enumerated. There is a possibility that the uracil desoxyriboside obtained from desoxyribonucleate is an artefact.

Comments—**Dr. Zamenhof** *(Columbia University)*: In the case of DNA from *Hemophilus influenzae* the DNA as isolated cannot be an artefact because it shows full biological (transforming) activity directly prior to chemical analysis.

Q.—**Dr. R. Gaudry** *(Laval University)*: Is anything known about the nature of the linkage between the nucleic acids and the carrier protein?

A.—**Dr. Butler:** All the evidence indicates that the linkage is of the salt or electrovalent type between the phosphate groups of the nucleate and the amino groups of the basic protein. But the size and complexity of the protein and nucleate components make it possible that other forces such as van der Waals forces may be important.

Q.—**Dr. S. G. A. Alivisatos** (*McGill University*): How does the glycosidic linkage at N_9 influence the dissociation of the amino groups of the purines, and how does the attachment to the pyrimidine component influence the dissociation of amino substituents?

A.—**Dr. Butler:** It has the effect of reducing the pK_a values of these amino groups, that is, they become weaker bases.

Q.—**Dr. E. Gordon Young** *(National Research Council, Halifax)*: What methods were used for the isolation of the nucleic acids?

A.—**Dr. Butler:** The gentlest methods of isolating nucleic acids are based on an initial isolation of the nucleoprotein and its subsequent deproteinization with strong salt solutions, chloroform emulsification, or sodium dodecyl sulphate.

BIOGENESIS OF THE NUCLEIC ACIDS[1]

By David Elwyn[2]

The biogenesis of the nucleic acids encompasses a large field much of which remains to be explored. A start has been made as to the metabolic origin of the various nucleic acid constituents, the nitrogenous bases, sugar, and phosphate. Much is known of the pathways of synthesis of the nucleotides and nucleosides but very little of the manner in which these molecules are combined to form the nucleic acids or nucleoproteins.

A number of methods have been used by investigators to explore these fields; the earlier workers used balance studies primarily. Later investigations fall into three main categories. Microbiological investigations, use of isolated enzyme systems, and studies involving isotopic tracers (34).

The present paper will deal primarily with the biosynthesis of the nitrogenous bases and their incorporation into the nucleosides and nucleotides as it occurs in higher animals (birds and mammals). A number of excellent review articles and symposia deal with this and other aspects of nucleoprotein synthesis (14, 15, 23, 34, 52, 54, 62).

Biosynthesis of Purines

It was early recognized that purines and pyrimidines were not essential dietary constituents and were synthesized by mammals and birds (52). The study of their synthesis was largely based on balance studies first with whole animals and later with tissue slices. The results of these experiments led to many misleading and conflicting conclusions. A large number of compounds, particularly urea, arginine, and histidine were at one time or another considered to be precursors of the purine ring. The work of Krebs and co-workers (8, 18, 43, 44) on hypoxanthine synthesis in pigeon liver slices indicated that ammonia, pyruvate, and other compounds might be involved, but it was only with the introduction of the isotopic tracer technique in this field that a real step forward was made as to the nature of the purine precursors.

Barnes and Schoenheimer (3) in 1943 fed N^{15}-labeled ammonium citrate and urea to pigeons, and isolated the excretory uric acid, the nitrogenous bases of the nucleic acids and several of the amino acids from the mixed internal organs (see Table I). These experiments showed that ammonia nitrogen was extensively incorporated into the ring of purines from both the excreta and the tissues. The low N^{15} values for the amino acids histidine, arginine, and glutamic and aspartic acids eliminated them as possible precursors of

[1] *Manuscript received January 27, 1953.*

Contribution from the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York.

The work from this laboratory was supported by grants from the American Cancer Society, upon recommendation of the Committee on Growth of the National Research Council, from the Lederle Laboratories Division of the American Cyanamid Company, and from the National Institutes of Health, United States Public Health Service.

[2] *Life Insurance Medical Research Postdoctoral Fellow, 1950–1952. Present address: Department of Biological Chemistry, Harvard Medical School, Boston, Mass.*

TABLE I

INCORPORATION OF N^{15} AMMONIUM CITRATE INTO PURINES OF THE PIGEON*

	Atom % excess N^{15} calculated on basis of 100% N^{15} in ammonium citrate given	
	Experiment 1**	Experiment 2***
Uric acid	15.5	
Guanine	6.3	
Adenine	5.5	2.7
Cytosine		3.1
Thymine		3.0
Amide nitrogen	6.2	
Glutamic acid	2.0	
Aspartic acid	2.7	
Arginine	0.7	
Histidine	0.6	

* *Data taken from Barnes and Schoenheimer (3).*
** *Three pigeons: 50 mgm. N per pigeon per day fed as ammonium citrate; duration of experiment four and one half days.*
*** *Six pigeons: 50 mgm. N per pigeon per day fed as ammonium citrate; duration of experiment three days.*

the purines. The low N^{15} values in the purines after the feeding of urea appeared to eliminate this compound also as a direct precursor of the purines. The authors concluded that the purines of the nucleic acids were constantly being synthesized and degraded in a manner similar to that of the internal organ proteins, and that the source of the ring nitrogen was the "biologically labile 'pool' of nitrogen which is involved in numerous interconversions in the body" (3). Other experiments by the same authors indicated that the pathway of purine synthesis in rats was similar to that in pigeons.

Additional evidence that arginine and histidine are not employed in purine synthesis was found by Bloch and Schoenheimer (10), and Tesar and Rittenberg (63), who administered N^{15}-labeled arginine and histidine to rats.

The work of Buchanan, Sonne, and Delluva (13, 58) in 1948 did much to further clarify the nature of purine precursors. These authors administered a number of C^{13}-labeled compounds to pigeons and isolated uric acid and respiratory carbon dioxide. The uric acid was degraded so that the concentration of C^{13} in each position could be determined. Some of the results are shown in Table II.

In all experiments including that in which labeled carbon dioxide was administered, the C^{13} value for carbon atom 6 of uric acid was very close to that for respiratory carbon dioxide, a strong indication that a new carbon dioxide fixation reaction had been discovered and that carbon dioxide was the immediate precursor of the 6 position.

After administration of carboxyl labeled acetate and formate a high isotope concentration was found in positions 2 and 8, the ureide carbons of uric acid.

TABLE II

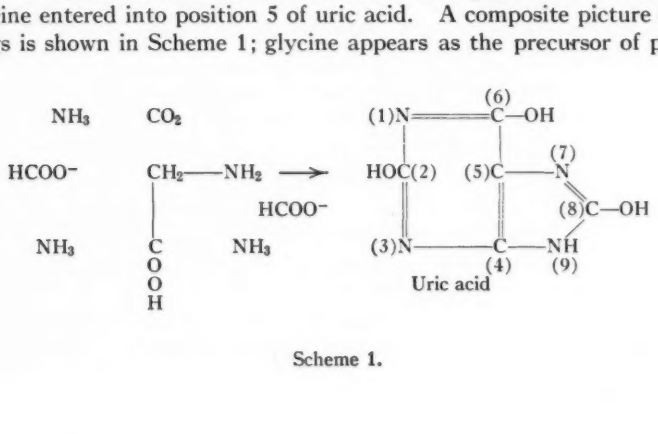
BIOLOGICAL PRECURSORS OF URIC ACID IN PIGEONS†

Precursor	Rate given (mM. per hr.)	Atom % excess C^{13} calculated on basis of 100% C^{13} in precursor					
		Uric acid carbon number					Respiratory CO_2
		2 + 8	4 + 5	4	5	6	
C*O₂††	0.75	0.2	0.5	0.9	0.0	3.1	3.4
HC*OOH‡	0.75	72	2.5			0.3	0.3
CH_3C^*OOH††	1.00	35	0.7	1.2	0.0	3.8	4.5
$NH_2CH_2C^*OOH$‡‡	0.50	0.0		22.7	2.7	2.1	2.3
dl-CH_3CHOHC^*OOH††	0.50	0.1		4.2	0.0	3.0	2.8
dl-$C^*H_3C^*HOHCOOH$††	0.50	1.9		1.3	2.6	1.6	2.0

† *Taken from data of Buchanan, Sonne, and Delluva (13, 58).*
†† *Experiments of 16–20 hr. duration.*
‡ *Experiment of 12 hr. duration.*
‡‡ *Experiment of 10 hr. duration.*

The results with acetate could not be confirmed (20, 33) and were reported in a later communication (56) to have been due to contamination of the acetate with formate. The results with formate were of particular interest since they showed for the first time that this compound could be extensively metabolized by higher animals.

When carboxyl labeled glycine was administered most of the isotope was found in the 4 position. When N^{15}-ammonium citrate was fed in conjunction with nonisotopic glycine it was found that the presence of glycine greatly lowered the utilization of ammonia for position 7. At about the same time it was shown by Shemin and Rittenberg (57) that glycine nitrogen was used for position 7 of uric acid in man, and Abrams, Hammarsten, and Shemin (1) showed the same for nucleic acid guanine of yeast. Shortly after these experiments were reported Karlsson and Barker (36) repeated much of Buchanan's work and extended it to find that in the pigeon the α-carbon atom of glycine entered into position 5 of uric acid. A composite picture of these findings is shown in Scheme 1; glycine appears as the precursor of positions



Scheme 1.

4, 5, and 7; positions 1, 3, and 9 are derived from ammonia or the labile nitrogen "pool", position 6 from carbon dioxide, and positions 2 and 8 from formate.

A large number of subsequent investigations have demonstrated that this pattern of synthesis does not apply solely to uric acid. Nucleic acid purines from yeast, rats, pigeons, and bacteria have been shown to be synthesized from similar precursors (4, 5, 9, 16, 17, 19, 20, 24, 33, 38, 40, 46, 61, 64). No evidence has been obtained that precursors not closely related to these participate in purine synthesis. It seems likely that most, if not all, organisms that are able to synthesize purines do so from the same or similar precursors.

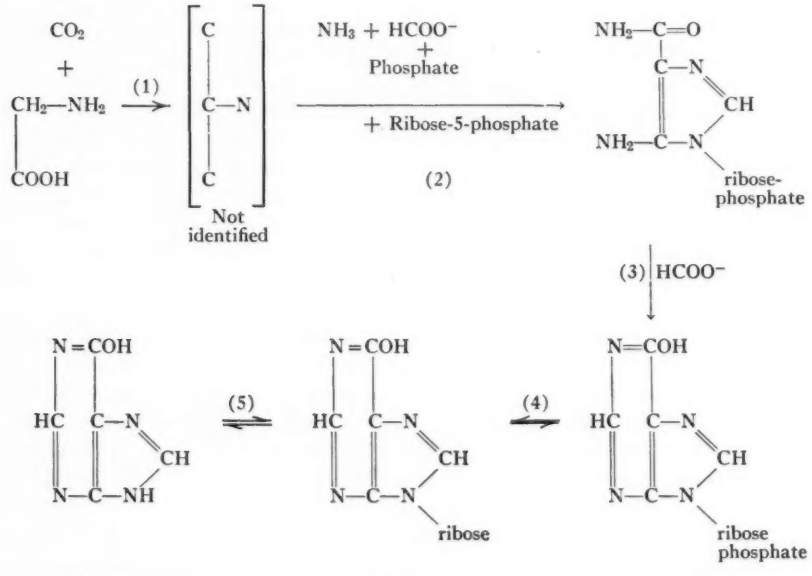
Of continuing interest is the nature of the direct precursor of the 2 and 8 positions. Unlike glycine and carbon dioxide, formate is present in the tissues of higher animals in very small quantities if at all. The discovery that administered formate was used in uric acid synthesis was followed by a number of investigations demonstrating that formate, methanol, and formaldehyde could be used by the rat for the synthesis of the labile methyl groups, the β-carbon of serine, and possibly other compounds. This has led to increasing interest in the actual nature of one-carbon intermediates in living organisms and in the exact nature of the precursor of the 2 and 8 positions of purines. Elwyn and Sprinson administered serine-3-C^{14} to pigeons (20) and rats (19, 21) and found that its β-carbon atom was used like formate for the 2 and 8 positions of uric acid, guanine, and adenine. Sprinson and Rittenberg (59) have shown that carbon atom 2 in the imidazole group of histidine is used in the same manner for uric acid and nucleic acid purines of pigeons. It seems probable that other sources of one-carbon intermediates such as the labile methyl groups would be utilized in a similar way. The exact nature of this one-carbon intermediate, or whether there are several, has still to be clarified.

The utilization of the carbon atom 2 of histidine may account for the stimulating effect of histidine for purine synthesis found in earlier experiments. It seems evident, however, that histidine as such is not a precursor of the purines, but rather that it, among other compounds, contributes to a one-carbon compound which in turn serves as a precursor of position 2 and 8 of the purine ring.

Greenberg (26, 27, 28, 29) has made important contributions towards clarifying the mechanism of purine synthesis. He used pigeon liver homogenates which synthesize hypoxanthine, and to these he added various radioactive precursors as glycine, carbon dioxide, and formate. He isolated hypoxanthine and demonstrated its synthesis from these precursors. In addition he was able to isolate and characterize a number of intermediates of hypoxanthine synthesis in this system by combining chromatographic, enzymological, and spectrophotometric techniques.

When glycine-1-C^{14} was used there was isolated an unidentified radioactive compound which was not found when radioactive formate was employed as the precursor. This indicates a reaction of glycine and carbon dioxide prior to

their reaction with formate. In experiments where formate was the precursor inosine and inosinic acid were isolated in addition to hypoxanthine. It was shown that the radioactivity of the inosinic acid was greater than that of inosine, which in turn was greater than that of hypoxanthine. In addition ribose-5-phosphate and compounds yielding ribose-5-phosphate stimulated the production of hypoxanthine. Greenberg was also able to show the reversibility of these steps in that radioactive hypoxanthine gave rise to inosine and inosinic acid. Greenberg concluded that 4-amino-5-imidazole carboxamide (which has been shown to give rise to hypoxanthine in this system (55)) was not on the direct pathway of purine synthesis, but that possibly its riboside was. A schematic representation of these results is shown in Scheme 2, as a five step reaction. It is evident that a number of these steps actually consist of a series of reactions which further research may be expected to clarify. It is significant, in view of the poor incorporation of dietary purines into nucleic acids, that the synthesis of hypoxanthine proceeds via the nucleotide.



Scheme 2.

Biosynthesis of Pyrimidines

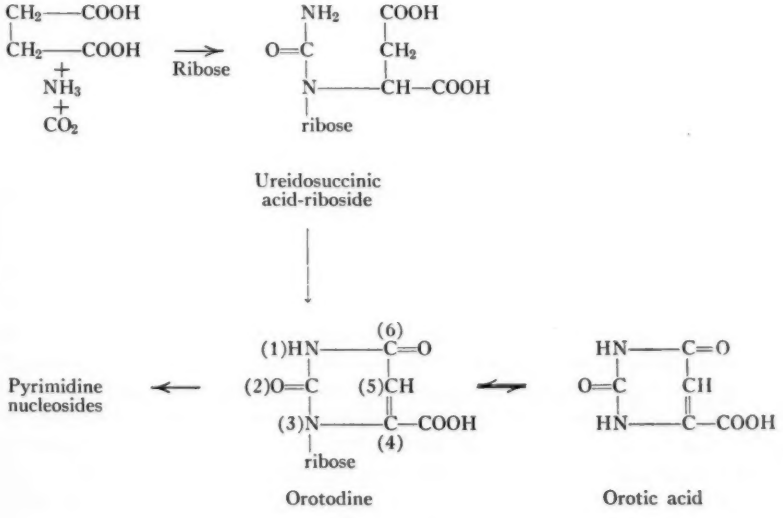
Much less work has been done on the synthesis of the pyrimidines. Barnes and Schoenheimer observed values of N^{15} as high as or higher in the pyrimidines than in the purines (Table I), after the feeding of labeled ammonium citrate,

indicating that the ring nitrogen of the pyrimidines was also derived from the metabolic "pool" in pigeons and rats. Glycine nitrogen has since been found to be incorporated into the pyrimidine ring (9).

Loring and Pierce (39) observed in 1944 that orotic acid could be substituted as a growth factor for uracil in certain *Neurospora* mutants. Mitchell and Houlahan (42) considered that orotic acid as such was not directly involved in pyrimidine synthesis and found evidence that oxalacetate, amino fumarate, and amino fumaric diamide could also be utilized for pyrimidine synthesis in *Neurospora*. Orotic acid has also been shown to be utilized by rats for pyrimidine synthesis (2, 66, 67). It is quite possible, however, that it is a derivative of orotic acid that is on the main pathway of pyrimidine synthesis, i.e., orotidine or orotidylic acid. Orotidine has, in fact, been recently isolated from *Neurospora* mutants.

Carbon dioxide has been shown to be incorporated into the 2 position of uracil in rats (33, 37). Recently Wilson and co-workers have reported that ureidosuccinic acid is used extensively for pyrimidine synthesis in *Lactobacillus vulgaris* (68).

A possible pathway of pyrimidine synthesis is shown in Scheme 3. There is little direct evidence for this scheme, as opposed to others, but it appears to be consistent with most of the data so far obtained. The incorporation of carbon dioxide into position 2 of uracil is consistent with this scheme. Lagerkvist, however, has found a significant incorporation of radioactive carbon dioxide into the 4 position of uracil in rats (37). The carboxyl carbon atom, but not the methylene carbons, of succinic acid or other four carbon acids in equilibrium with the Krebs cycle components, would be expected to be labeled



Scheme 3.

after administration of radioactive carbon dioxide. However, according to the above scheme, the 4 position of orotic acid is derived from the methylene carbon of succinic acid, while the 6 position is derived from the carboxyl. Lagerkvist found little if any radioactivity in the 6 position, while the 4 position was as active as the 2. In Lagerkvist's degradation of uracil the 2, 5, and 6 carbons were measured directly, the 4 carbon by difference, a method involving large errors. It would be desirable before attempting to reconcile this with other findings to repeat it by direct measurement of the isotope concentration in the 4 position.

Thymine, 5-methyl cytosine, and 5-hydroxymethyl cytosine (69) differ from the other pyrimidine constituents of nucleic acids by the presence of an additional carbon atom attached to position 5 of the ring. Elwyn and Sprinson (19) investigated the biological origin of the methyl group by feeding serine-3-C^{14} and glycine-2-C^{14} to rats and isolating and degrading the nucleic acid thymine. It was found that the β-carbon atom of serine was incorporated extensively into the methyl group of thymine, the radioactivity of the latter being close to that of the 2 and 8 positions of guanine from the same experiments. The α-carbon of glycine was incorporated to a much less extent. In both cases 90% of the radioactivity of the thymine was accounted for by the methyl group, indicating a methylation of a pyrimidine ring or ring precursor, rather than an incorporation of serine as a whole into the ring, since α-labeled glycine gives rise to α, β-labeled serine (53) and in the latter case would be expected to label the pyrimidine ring. An experiment in which serine labeled with both C^{14} and deuterium was used (22) indicated that carbon–hydrogen bonds remain intact during the transformation of the hydroxymethyl group of serine to the methyl group of thymine. This appears to eliminate formate or its derivatives as one-carbon intermediates in this reaction, although formate is known to be used for thymine methyl group synthesis in the rat (64).

Incorporation of Larger Molecules into Nucleic Acids

While it has been shown that preformed purines and pyrimidines are not dietary essentials, and that most organisms synthesize these from simple metabolic precursors, there has been nevertheless a continuing interest in the metabolic reactions of the breakdown products of nucleic acids, the nucleotides, nucleosides, and free bases. In addition certain compounds not found as constituents of nucleic acids but apparently involved in their synthesis, such as orotic acid and 4-amino,5-imidazole carboxamide have concerned investigators in this field. The apparent uniformity of synthesis of purines and pyrimidines in widely different species does not apply to the manner in which organisms react to preformed purines, pyrimidines, and their derivatives. Here there are widespread species differences and conclusions cannot be carried from one species to the next. This discussion will deal primarily with the laboratory rat since it is the most widely investigated animal.

Plentl and Schoenheimer (45) fed N^{15}-labeled guanine, thymine, and uracil to rats and isolated purines and pyrimidines from the nucleic acids. Although

the compounds were absorbed and catabolized, they were not incorporated into nucleic acids to an appreciable extent. Subsequently Brown and co-workers (11, 24, 25) administered labeled adenine and cytosine to rats. Cytosine, like guanine, thymine, and uracil, was not incorporated into the nucleic acids. Adenine on the other hand was extensively incorporated into nucleic acid adenine and guanine. In adult rats there was high incorporation into the PNA of the liver but practically none into the DNA. In rats with regenerating livers there was a roughly equal incorporation into both DNA and PNA. The isotope level of the PNA fell off after administration of labeled adenine was stopped. In rats with regenerating liver the level in the DNA remained almost constant for considerable periods after adenine administration and growth had ceased. The bearing of these findings on the rate of turnover of nucleic acids will be discussed later, but it is noteworthy that adenine is the only one of the nucleic acid bases incorporated into nucleic acids in the rat. Moreover, the behavior of adenine is quite different from that of other precursors of nucleic acid purines, such as formate, glycine, serine, or ammonia. The ratio of incorporation PNA : DNA is as high as 29 : 1 when adenine is the precursor, but ranges from 1 : 1 to 4 : 1 when other precursors are employed. While adenine is incorporated to a much greater extent than other precursors it is difficult to evaluate the significance of this finding since compounds such as formate and glycine participate in many metabolic reactions.

Furst and Brown (24) investigated the possibility that the different results obtained with adenine and other precursors were due to variation in experimental conditions and techniques. They administered simultaneously C^{14}-labeled adenine and N^{15}-labeled glycine to adult rats and rats with regenerating livers and were able to confirm the previous findings.

As a general rule the free bases are not involved in nucleic acid synthesis. There is no evidence that they exist in appreciable amounts in mammalian tissues or diets. The contrast in behavior of adenine to other purine precursors would indicate that adenine is not an exception but that it is incorporated by a pathway different from the normal synthesis of purines. Dietary nucleic acids appear to be broken down in the gut to nucleotides and nucleosides and absorbed as such. The contrasting behavior of adenine to guanine and the pyrimidines may be related to facts disclosed by certain enzymatic studies. Kalckar (35) has described a rat liver enzyme which catalyzes the reversible reaction:

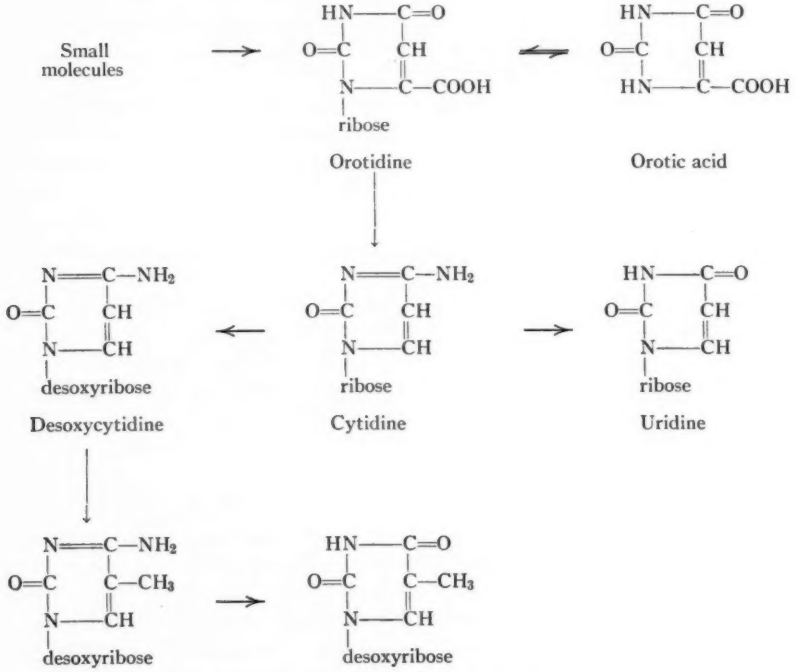
$$\text{purine} + \text{ribose-1-phosphate} \rightleftharpoons \text{purine-riboside} + \text{phosphate}.$$

The equilibrium favors nucleoside synthesis. Only guanine and hypoxanthine react in this system, adenine does not. Richert and Westerfeld (48) have shown that rat liver homogenates oxidize guanine, hypoxanthine, xanthine, and their nucleotides and nucleosides to uric acid. Adenosine and adenylic acid react in this system but adenine does not. One might speculate that these enzymes function primarily in purine catabolism, and that their inactivity with respect to adenine permits appreciable quantities of the latter to participate in nucleic acid synthesis.

Bendich and co-workers (6, 7) have studied the mechanism of the conversion of adenine to guanine. In testing a number of possible intermediates they found that N^{15}-labeled 2,8-diamino purine was converted to PNA guanine of rat internal organs, but not to adenine. They postulate that adenine is aminated at the 2 position to give 2,6-diamino purine which is subsequently converted to guanine. Since free guanine and guanosine are not incorporated, the 2,6-diamino purine must first be converted to the nucleotide or other derivative before hydrolysis of the 6-amino group takes place. 2,6-Diamino purine, which has not been shown to occur in nature, is the only purine other than adenine known to participate in nucleic acid synthesis.

None of the pyrimidine constituents of nucleic acids is utilized for nucleic acid synthesis in rats. The only pyrimidine known to participate is orotic acid, which has already been discussed. It is perhaps significant that orotic acid, unlike other pyrimidines, is known to occur in the free form, namely in milk.

The behavior of nucleosides and nucleotides differs markedly from that of the free bases. The pyrimidine nucleosides have been particularly well studied. Hammarsten and Reichard (31) and Reichard and Estborn (47)



Scheme 4.

administered N^{15}-ring labeled uridine, cytidine, desoxycytidine, and thymidine to rats and then isolated the purine and pyrimidine constituents of the internal organs. Uridine was used poorly and in a nonspecific way, i.e., the N^{15} values for the purines were nearly as high as for the pyrimidines. Cytidine was incorporated extensively into the cytosine and uracil of PNA, and to a lesser but significant extent into the cytosine and thymine of DNA. Hammarsten and Reichard suggested that since the latter involves a change in the sugar moiety, the 2 position of ribose in cytidine is reduced to give desoxycytidine. When desoxycytidine was fed it was incorporated into the cytosine and thymine of DNA, but not incorporated into PNA. Thymine in the form of its desoxyriboside was likewise incorporated only into the thymine (but not the cytosine) of DNA. The interconvertibility of these nucleosides and the role of cytidine in particular suggest that all of the pyrimidines may be derived from a common precursor. Since the free bases are not utilized this precursor may be a nucleoside or nucleotide. A possible scheme of pyrimidine synthesis taking into account these facts is shown in Scheme 4. However, many alternate schemes are equally feasible. It is possible that 5-hydroxymethyl cytosine (69), or a derivative of it, might be an intermediate in the methylation of the cytosine ring, although this compound is not known to occur in rats.

Roll (49) has recently tested N^{15}-labeled cytidylic and uridylic acids, and has found their behavior in the rat to be almost identical to that of cytidine and uridine.

Guanosine (31) and desoxyhypoxanthosine (47), the only purine nucleosides so far tested, are not efficient precursors of the nucleic acids. The nucleotides, guanylic and adenylic acids, are well utilized however. Roll and Weliky (50) fed N^{15}-labeled adenylic and guanylic acids to rats and isolated the purines and pyrimidines from liver PNA and DNA. When adenylic acid was fed there was a high incorporation into adenine and guanine of PNA. With guanylic acid there was incorporation of isotope in the guanine only, not more than a trace of isotope being found in the DNA purines or in any of the pyrimidines. Previous experiments (51) had shown that N^{15}-labeled yeast nucleic acid is a somewhat less effective precursor than the nucleotides.

Turnover Rates—The rate of turnover of the nucleic acids, particularly DNA, has attracted considerable interest in the last few years. Barnes and Schoenheimer drew the conclusion from their work with N^{15}-ammonium citrate that nucleic acids, like proteins, were being constantly degraded and synthesized. A number of subsequent investigations have indicated that this conclusion is valid only for PNA, and that DNA may be relatively inert, undergoing synthesis and breakdown only during cell division. Results of other investigations have appeared to conflict with this concept of the biochemical stability of DNA and there remains considerable uncertainty on this question. The situation is complicated by the fact that many of the results are derived from experiments not primarily concerned with measurement of turnover rates, and that few of those which have been so concerned have included sufficient controls to permit of more than tentative conclusions.

Experiments designed to measure the rate of turnover of DNA, to be conclusive, should meet the following requirements. (1) During administration of the isotopic compound the isotope level of the immediate precursors of DNA should be estimated. (2) Measurements of isotope incorporation into DNA should be made at successive time intervals. (3) After isotope administration has ceased, the amount of loss of isotope from DNA should be measured at successive time intervals. (4) The rate of new cell formation in the tissue or tissues concerned should be estimated or preferably measured.

A number of experiments on the incorporation of radioactive phosphate into the nucleic acids of various tissues of the rat have been made (12, 30, 41, 60), which are in general agreement that there is a much lower incorporation of isotope into liver DNA than PNA, and that DNA incorporation can be closely correlated with estimates or measurements of mitotic rates in different tissues. Hammarsten and Hevesy (30) injected radioactive phosphate into rats, killed the rats two hours after injection, and determined the specific activities of the PNA, DNA, and inorganic phosphate of the liver, spleen, and intestinal mucosa. On the assumption that the immediate precursor of PNA and DNA phosphate is the inorganic phosphate, they were able to estimate the rate of renewal of DNA and PNA phosphate. A summary of their results is shown in Table III. PNA is renewed at a rapid rate not only in intestine and spleen, in which there is a rapid rate of new cell formation, but also, although to a less extent, in liver, in which very little cell proliferation takes place. In the case of DNA there is a marked contrast between the renewal in liver and other organs. Stevens, Daoust, and Leblond (60) in their paper delivered at this symposium have reported results consistent with these and have also measured the rate of mitosis in liver (0.67% per day) and intestinal mucosa (54% per day) of rats. Brues, Tracy, and Cohn (12) found that P^{32} once incorporated into resting or regenerating liver was retained for periods up to 13 days. These data are consistent with the thesis that DNA renewal occurs only during cell division and demonstrate the markedly different rates of renewal in different tissues.

Volkin and Carter (65) injected rats with radioactive phosphate and isolated the individual nucleotides from PNA and DNA of resting liver.

TABLE III

RATE OF RENEWAL OF PNA AND DNA PHOSPHATE IN RAT ORGANS

Organ	Specific activity of nucleic acid phosphate as per cent of inorganic phosphate		Ratio of incorporation PNA : DNA
	PNA	DNA	
Liver	3.45	0.105	33 : 1
Spleen	6.65	2.2	3 : 1
Intestine	5.6	2.85	2 : 1

They found, contrary to the results cited above, approximately equal incorporation of isotope into PNA and DNA. In these experiments the specific activity of the inorganic phosphate was not determined, and no estimate of the absolute turnover rate of the nucleic acids could be made. Because of differences in experimental procedure it is impossible to determine the reasons for these conflicting results. Further, more adequate studies of phosphorus turnover would appear necessary to resolve these conflicts. This investigation also demonstrated a marked difference in incorporation of radioactive phosphate into the various nucleotides. Thymidylic acid contained twice as much isotope as the other DNA nucleotides. Adenylic acid contained 50% more isotope than the other PNA nucleotides. These results indicate considerable heterogeneity with respect to phosphorus turnover within both nucleic acids, a phenomenon found also with other nucleic acid components.

The investigations of Brown and co-workers (5, 11, 24, 25) of the incorporation of labeled adenine into rat nucleic acids has already been discussed. Adenine was incorporated into the nucleic acids of all tissues, the greatest incorporation occurring in the liver and less in the spleen and intestine. The liver PNA isotope values indicated as much as 22% new synthesis over a five day period, in agreement with the general conclusion that PNA has a rapid turnover. The DNA isotope values indicate only 0·29% synthesis in the same period, which would be consistent with the amount of new cell proliferation. The use of net isotope incorporation as a measure of synthesis is valid when adenine is a precursor, since it has been shown that administered adenine undergoes practically no dilution in the rat (6). In the pooled viscera they found a similar PNA : DNA ratio of 29 : 1. This ratio and the higher incorporation into liver PNA are in marked contrast to the findings of Hammarsten and Hevesy that phosphate gives a PNA : DNA ratio of 2 : 1 for the whole animal and is incorporated better into spleen and intestine than into liver.

A large number of investigations involving other purine and pyrimidine precursors (5, 9, 19, 22, 24, 31, 32, 38, 46, 59, 64) have yielded PNA : DNA ratios varying from 1 : 1 to 4 : 1 for various organs (including liver), for mixed viscera, and for tumors. Because of the marked difference between organs, the data for mixed viscera are difficult to interpret. In most of the investigations with liver (9, 24, 38, 59), the isotope levels were measured at only one time interval after administration of the precursor. In addition, since the precursors employed, e.g. glycine or formate, are metabolically active, no estimate of the isotope level of the immediate precursors of the nucleic acid constituents could be made. As a result no estimate of turnover rates can be made, for the low PNA : DNA ratios obtained could be due to much lower incorporation into PNA or higher incorporation into DNA, as compared to adenine.

Bendich, in one investigation with formate (5), has measured the isotope level at two time intervals, 1 and 24 days after the last formate injection. He was able to calculate the retention of isotope over the 23 day period.

A summary of his data for resting and regenerating rat liver is compared to similar data for adenine in Table IV. The results with formate are typical of other experiments with small molecule precursors and serve as a good example of the contrast in the behavior of these compounds to that of adenine. The major difference is found in the ratio of incorporation PNA : DNA in resting liver which is 4 : 1 for formate and 73 : 1 for adenine. Both the PNA : DNA ratio and isotope retention in regenerating liver are similar for both compounds. Unfortunately isotope retention studies have not been made with adenine for resting liver. The results with formate point up a marked distinction between isotope retention in resting and regenerating liver for DNA, the average value for the purines being 24% for resting and 63% for regenerating liver during the 23 day period. In the case of PNA, isotope is lost rapidly in both forms of tissue.

The difficulties of arriving at turnover rates without complete kinetic studies are illustrated by the results with formate in resting liver. If relative turnover rates are taken from the ratios of incorporation, PNA has four times the turnover rate of DNA; if they are taken from the percentage isotope retention the rate is only 1.5 times as high.

Bendich's experiments also demonstrated marked metabolic differences between the various purines and pyrimidines within the same nucleic acid. In addition two DNA fractions were obtained which exhibited different rates of incorporation and retention of isotope.

It is to be expected that more adequate studies of nucleic acid turnover will be forthcoming in the near future. One of the major reasons for the lack of such experiments to date has been that the necessary knowledge of the

TABLE IV

RETENTION OF ISOTOPE AND RATIO OF ISOTOPE INCORPORATION PNA : DNA IN RESTING AND REGENERATING RAT LIVER

Precursor	Isolated compound	Per cent retention in				Ratio of incorporation PNA : DNA*	
		Resting liver		Regenerating liver			
		PNA	DNA	PNA	DNA	Resting liver	Regenerating liver
C^{14} Formate**	Thymine		53		88		
	Adenine	10	15	7	56	3.6 : 1	1.2 : 1
	Guanine	13	33	13	69	4.0 : 1	1.2 : 1
N^{15} Adenine***	Total purines			15	74		1.4 : 1
	Adenine					73 : 1	1.4 : 1
	Guanine						2.5 : 1

* *PNA : DNA ratio calculated from isotope values for first day after precursor administration ended.*

** *Taken from Bendich (5). Per cent retention calculated from isotope values 1 and 24 days after last injection of formate.*

*** *Taken from Furst, Roll, and Brown (25). Per cent retention calculated from isotope values 1 and 21 days after adenine administration ended.*

precursors of nucleic acid components has only recently been acquired. Further clarification of the heterogeneous nature of nucleic acids, both structurally and metabolically, may be expected to result from these investigations.

While there are still large gaps in our knowledge of nucleic acid turnover, some general conclusions from present information seem warranted. PNA appears under all conditions to be rapidly renewed and degraded. The concept of DNA as biochemically stable is applicable, if at all, only to those tissues in which cell proliferation is small. The thesis that DNA synthesis and degradation occurs only or primarily during cell division is well supported, although there are some conflicting data not yet reconciled with this view.

References

1. ABRAMS, R., HAMMARSTEN, E., and SHEMIN, D. J. Biol. Chem. 173 : 429. 1948.
2. ARVIDSON, H., ELIASSON, N. A., HAMMARSTEN, E., REICHARD, P., VON UBISCH, H., and BERGSTRÖM, S. J. Biol. Chem. 179 : 169. 1949.
3. BARNES, F. W. and SCHOENHEIMER, R. J. Biol. Chem. 151 : 123. 1943.
4. BEN-ISHAI, R., VOLCANI, B., and BERGMANN, E. D. Arch. Biochem. 32 : 229. 1951.
5. BENDICH, A. Exptl. Cell Research Suppl. 2 : 181. 1952.
6. BENDICH, A., BROWN, G. B., PHILLIPS, F. S., and THIERSCH, J. B. J. Biol. Chem. 183 : 267. 1950.
7. BENDICH, A., FURST, S. S., and BROWN, G. B. J. Biol. Chem. 185 : 423. 1950.
8. BENZINGER, T. and KREBS, H. A. Klin. Wochschr. 12 : 1206. 1933.
9. BERGSTRAND, A., ELIASSON, N. A., HAMMARSTEN, E., NORBERG, B., REICHARD, P., and VON UBISCH, H. Cold Spring Harbor Symp. Quant. Biol. 13 : 22. 1948.
10. BLOCH, K. and SCHOENHEIMER, R. J. Biol. Chem. 138 : 167. 1941.
11. BROWN, G. B., ROLL, P. M., PLENTL, A. A., and CAVALIERI, L. F. J. Biol. Chem. 172 : 469. 1948.
12. BRUES, A. M., TRACY, M. M., and COHN, W. E. J. Biol. Chem. 155 : 619. 1944.
13. BUCHANAN, J. M., SONNE, J. C., and DELLUVA, A. M. J. Biol. Chem. 173 : 81. 1948.
14. CHRISTMAN, A. A. Physiol. Revs. 32 : 303. 1952.
15. COLD SPRING HARBOR SYMPOSIA on Quant. Biol. 12. 1947. Nucleic Acids and Nucleoproteins.
16. DRYSDALE, G. R., PLAUT, G. W. E., and LARDY, H. A. J. Biol. Chem. 193 : 533. 1951.
17. EDMONDS, M., DELLUVA, A. M., and WILSON, D. W. J. Biol. Chem. 197 : 251. 1952.
18. EDSON, N. L., KREBS, H. A., and MODEL, A. Biochem. J. 30 : 1380. 1936.
19. ELWYN, D. and SPRINSON, D. B. J. Am. Chem. Soc. 72 : 3317. 1950.
20. ELWYN, D. and SPRINSON, D. B. J. Biol. Chem. 184 : 465. 1950.
21. ELWYN, D. and SPRINSON, D. B. Unpublished observation.
22. ELWYN, D., WEISSBACH, A., and SPRINSON, D. B. J. Am. Chem. Soc. 73 : 5509. 1951.
23. EXPTL. CELL RESEARCH Suppl. 2. 1952. The chemistry and physiology of the nucleus.
24. FURST, S. S. and BROWN, G. B. J. Biol. Chem. 191 : 239. 1951.
25. FURST, S. S., ROLL, P. M., and BROWN, G. B. J. Biol. Chem. 183 : 251. 1950.
26. GREENBERG, G. R. Arch. Biochem. 19 : 337. 1948.
27. GREENBERG, G. R. Federation Proc. 8 : 202. 1948.
28. GREENBERG, G. R. Federation Proc. 9 : 179. 1949.
29. GREENBERG, G. R. J. Biol. Chem. 190 : 611. 1951.
30. HAMMARSTEN, E. and HEVESY, G. Acta Physiol. Scand. 11 : 335. 1946.
31. HAMMARSTEN, E. and REICHARD, P. Acta Chem. Scand. 4 : 711. 1950.
32. HAMMARSTEN, E., REICHARD, P., and SALUSTE, E. J. Biol. Chem. 183 : 105. 1950.
33. HEINRICH, M. R. and WILSON, D. W. J. Biol. Chem. 186 : 447. 1950.
34. KALCKAR, H. M. Harvey Lectures, Ser. 45. p. 11. 1949–50.
35. KALCKAR, H. M. J. Biol. Chem. 167 : 477. 1947.
36. KARLSSON, J. L. and BARKER, H. A. J. Biol. Chem. 177 : 597. 1949.

37. LAGERKVIST, U. Acta Chem. Scand. 4 : 1151. 1951.
38. LEPAGE, G. A. and HEIDELBERGER, C. J. Biol. Chem. 188 : 593. 1951.
39. LORING, H. S. and PIERCE, J. G. J. Biol. Chem. 153 : 61. 1944.
40. MARSH, W. H. J. Biol. Chem. 190 : 633. 1951.
41. MARSHAK, A. J. Cellular Comp. Physiol. 32 : 381. 1948.
42. MITCHELL, H. K. and HOULAHAN, M. B. Federation Proc. 6 : 506. 1947.
43. ÖSTRÖM, A., ÖSTRÖM, M., and KREBS, H. A. Biochem. J. 33 : 990. 1939.
44. ÖSTRÖM, A., ÖSTRÖM, M., KREBS, H. A., and EGGLESTON, L. V. Biochem. J. 33 : 995. 1939.
45. PLENTL, A. A. and SCHOENHEIMER, R. J. Biol. Chem. 153 : 203. 1944.
46. REICHARD, P. Acta Chem. Scand. 3 : 422. 1949.
47. REICHARD, P. and ESTBORN, B. J. Biol. Chem. 188 : 839. 1951.
48. RICHERT, D. A. and WESTERFELD, W. W. J. Biol. Chem. 184 : 203. 1950.
49. ROLL, P. M. Unpublished observation. See Bendich (5).
50. ROLL, P. M. and WELIKY, I. Federation Proc. 10 : 238. 1951.
51. ROLL, P. M., BROWN, G. B., DE CARLO, F. J., and SCHULTZ, A. S. J. Biol. Chem. 180 : 333. 1949.
52. ROSE, W. C. Physiol. Revs. 3 : 544. 1923.
53. SAKAMI, W. J. Biol. Chem. 178 : 519. 1949.
54. SCHLENK, F. Advances in Enzymol. 9 : 455. 1949.
55. SCHULMAN, M. P. and BUCHANAN, J. M. J. Biol. Chem. 196 : 513. 1952.
56. SCHULMAN, M. P., SONNE, J. C., and BUCHANAN, J. M. J. Biol. Chem. 196 : 499. 1952.
57. SHEMIN, D. and RITTENBERG, D. J. Biol. Chem. 167 : 875. 1947.
58. SONNE, J. C., BUCHANAN, J. M., and DELLUVA, A. M. J. Biol. Chem. 173 : 69. 1948.
59. SPRINSON, D. B. and RITTENBERG, D. J. Biol. Chem. 198 : 655. 1952.
60. STEVENS, C. E., DAOUST, R., and LEBLOND, C. P. This journal.
61. SUTTON, W. B., SCHLENK, F., and WERKMAN, C. H. Arch. Biochem. and Biophys. 32 : 85. 1951.
62. SYMPOSIA, SOC. EXPTL. BIOL. 1947. Nucleic acid.
63. TESAR, C. and RITTENBERG, D. J. Biol. Chem. 170 : 35. 1947.
64. TOTTER, J. R., VOLKIN, E., and CARTER, C. E. J. Am. Chem. Soc. 73 : 1521. 1951.
65. VOLKIN, E. and CARTER, C. E. J. Am. Chem. Soc. 73 : 1519. 1951.
66. WEED, L. L., EDMONDS, M., and WILSON, D. W. Proc. Soc. Exptl. Biol. Med. 75 : 192. 1950.
67. WEED, L. L. and WILSON, D. W. J. Biol. Chem. 189 : 435. 1951.
68. WRIGHT, L. D., MILLER, C. S., SKEGGS, H. R., HUFF, J. W., WEED, L. L., and WILSON, D. W. J. Am. Chem. Soc. 73 : 1898. 1951.
69. WYATT, G. R. and COHEN, S. S. Nature, 170 : 1072. 1952.

DISCUSSION

Q.—**Dr. C. Stevens** *(McGill University)*: Can one generalize from isotopic studies on one species as to the metabolism of nucleic acids in other species?

A.—**Dr. Elwyn** (*Columbia University*): No. But sufficient work has been done on a number of widely different species to enable one to generalize on the nature of the small molecule precursors of the purines. The reactions involving preformed bases and the more complex molecules vary considerably from one species to another.

Q.—**Dr. G. R. Wyatt** *(Insect Pathology Laboratory, Sault Ste. Marie, Ont.)*: Is the conversion of ribose to desoxyribose a one-step reaction?

A.—**Dr. Elwyn:** Reichard and co-workers have suggested that the conversion of cytidine to desoxycytidine occurs by reduction at the 2-position of

ribose; however, the evidence is not conclusive. It is possible that desoxyribose replaces ribose in an exchange reaction which works with cytidine but not with cytosine.

Q.—**Dr. D. Beall:** To what extent are the purine and pyrimidine bases of the food utilized in the body and may these bases be modified by the microorganisms in the intestine?

A.—**Dr. Elwyn:** I don't know very much about this subject, but perhaps the comparatively small utilization of ingested yeast nucleic acid compared with that of injected nucleotides may be accounted for by bacterial deamination or other degradations.

Q.—**Dr. Gaudry:** Is there any evidence that amino acids other than glycine, serine, and histidine are involved in the synthesis of the purine and pyrimidine bases?

A.—**Dr. Elwyn:** Ureidosuccinic acid, which is a derivative of aspartic acid, is utilized by *Lactobacillus vulgaris* for the synthesis of uracil. Whether aspartic acid itself is involved has not been reported. I am not aware that other amino acids have been shown to be directly involved in the synthesis of these bases. The imidazole carbon of histidine is utilized for the synthesis of serine and the methyl groups of choline and may be expected to contribute to the formation of the methyl group of thymine, although no direct evidence for this has been reported.

Q.—**Dr. W. W. Hawkins** *(National Research Council, Halifax)*: Is there any evidence that ammonia nitrogen is utilized in the synthesis of the ring structures of the purine and the pyrimidine bases?

A.—**Dr. Elwyn:** Apart from the incorporation of the nitrogen of glycine into position-7 of the purine molecule little is known about the immediate sources of the nitrogen in the purines and pyrimidines.

BIOLOGICAL ACTIVITY OF THE NUCLEIC ACIDS[1]

By Stephen Zamenhof, Hattie E. Alexander, and Grace Leidy

Abstract

The biological activities of the pentose (PNA) and desoxypentose nucleic acids (DNA) are discussed. Bacterial PNA inhibit certain streptococcal desoxyribonucleases. Bacterial DNA are known to act as transforming principles or heredity determinants. Methods are described for the isolation and purification of DNA from *Hemophilus influenzae* active as transforming principles in concentrations $3 \times 10^{-4}/\gamma$cc. The inactivation by heat, changes of pH and ionic strength, desoxyribonuclease, and formaldehyde is studied.

Introduction

The ubiquity of nucleic acids in nature makes imperative an attempt to answer the intriguing question: What are their roles in the living systems? That there is more than one role one can easily guess, if only on the basis of the dichotomy in the chemistry of nucleic acids—we mean here the existence of the two major groups: the pentose (PNA) and desoxypentose nucleic acids (DNA). But one cannot assume a priori that the nucleic acids within each group serve but one purpose. Thus in a cell one PNA is situated in the nucleolus (28, 34), another PNA in the chromosomes (28), a third one in the mitochondria, a fourth in submicroscopical particles, a fifth soluble in cytoplasm (27), and sometimes a sixth in the Gram-positive layer (22). This wide variety as well as the differences in chemical compositions of these various PNA (in few cases when these were isolated and analyzed separately) (18, 16, 37, 32) make it highly improbable that they would all serve but one purpose. As a matter of fact we cannot even assume that the PNA isolated from one cell fraction serves only one purpose. In the case of crystalline viruses the situation is simpler in that indeed only one fraction of PNA seems to be present and this fraction is thought to be connected with the transmission of hereditary properties as mentioned later. However, even here there is no proof that this fraction has but one role.

The situation for the cellular DNA is more fortunate than the cellular PNA, and resembles the PNA of crystalline virus. The DNA is usually situated in only one part of the cell, namely, the chromosomes, and has been isolated as a substance monodisperse (10, 13, 15, 43, 20, 41) in electrophoresis and ultracentrifuge studies: it has been also postulated (10, 1, 2) that the DNA has a role of the heredity determinant. However, the reservations mentioned in connection with the crystalline viruses apply also to DNA. This subject will be discussed fully later.

It is of interest to note that recently DNA has been unquestionably found also outside of the chromosomes, namely in the egg cytoplasm (for a recent

[1] *Manuscript received January 27, 1953.*

Contribution from the Department of Biochemistry and the Department of Pediatrics, College of Physicians and Surgeons, Columbia University, and Babies Hospital, New York. This work has been supported by research grants from the National Institutes of Health, United States Public Health Service.

work on this subject see (17)). In one case, when such a DNA had been analyzed for the content of the individual purines and pyrimidines (19), it was found to be in this respect very similar to the DNA of the cell nuclei (14). Whether this DNA has a specific function or whether it represents merely a nutrient or an incidental product of destruction of some of the egg-producing gland cells or egg-nourishing cells, cannot be said at present.

In this lecture, time will not permit us to go into the problem of biological activity of simple building units of nucleic acids such as nitrogenous bases, nucleosides, or nucleotides, or of various degradation products of nucleic acids. What we will try to discuss here are the biological actions of the so-called "native" nucleic acids, presumably in the state somewhat similar to the one in which they exerted their activities in the living systems; moreover, the activities under discussion are such that they disappear upon denaturation of the nucleic acids. Such roles, well supported by sound experimental evidence, are few as compared with the wide list of possibilities, but some of them are extremely important, especially with reference to DNA. We will not discuss here the speculative roles, such as the role as energy donors or in connection with protein synthesis, for which the unequivocal experimental evidence is still missing.

Since we are going to talk about the so-called "native" nucleic acids, a definition of this term is obviously due. Actually, the term "native" with reference to nucleic acids extracted from the cell is misleading because the extraction and purification procedures are designed to destroy the bonds anchoring the nucleic acids to their prosthetic compounds, that is proteins, thus altering the native state of both components. Nevertheless, carefully isolated nucleic acids may exhibit certain characteristic features, such as biological activity (transforming activity), which irreversibly disappears upon mistreatment and which therefore must have been present in the original molecule in the cell. Merely because of the lack of better definition, such a carefully isolated nucleic acid may be considered to have certain "native" properties and any change (other than depolymerization) which destroys the "native" properties may be called "denaturation", in conformity with the nomenclature proposed for proteins (33). Briefly, to determine whether a nucleic acid preparation is native may be actually impossible, but it is often very easy to show that it has been denatured.

The Reported Biological Activity of the Pentose Nucleic Acids

The first point in our program will be the reported biological activity of the pentose nucleic acids. In the field of cellular pentose nucleic acid the progress has been extremely slow, paralleling slow progress in the field of their chemistry. This is partly due to the previously mentioned difficulty in obtaining the PNA as one chemical entity from just one part of the cell; another reason is the difficulty in avoiding denaturation when deproteinizing the PNA which is usually more firmly bound to the protein than is the DNA. Indeed, only one bona fide biological activity has been reported for cellular

PNA, in which various PNA's have not been separated or deproteinized. Accordingly, it would be of course very wrong to assume that this biological activity of PNA is the only one or even the important one.

The biological activity referred to here is the phenomenon discovered by Bernheimer (11, 12). He found that the crude bacterial extracts are capable of inhibiting the desoxyribonuclease of some of the streptococcal strains. The inhibitor was resistant to the action of proteolytic enzymes or protein denaturing agents ($CHCl_3$) but was easily destroyed by even very low concentrations of pancreatic ribonuclease or intestinal phosphatases. These results are taken as a proof of the intact PNA, rather than the protein, nature of the inhibitor. This inhibitory action is specific in that only the desoxyribonuclease of Group A streptococci is inhibited, whereas the ones from Groups *B* or *C* streptococci or from yeast (39), barley (39), or pancreas are not affected. Conversely, the desoxyribonuclease inhibitor from yeast (39), or ribonucleic acid fractions from yeast, higher plants, or mammalian tissue had no effect on streptococcal desoxyribonuclease. It is interesting to note that the intact polyribophosphate (42), a specific immunologically active substance of Type *b* *Hemophilus influenzae*, which seems to be similar to the ribose-phosphate chain of ribonucleic acid, also had some (although smaller) inhibitory effect on streptococcal desoxyribonuclease.

As to the other roles of PNA, one may speculate about its role in crystalline viruses as the heredity determinants in these low species; the evidence is, however, a very indirect one. Since some of these viruses contain nothing else but the ribonucleoprotein, then obviously ribonucleoprotein is sufficient to determine all the hereditary properties of these species. This action could be due to the ribonucleic acid alone, or to the protein alone, or to the mode of combination of the two. By analogy with the DNA, where good evidence exists that the DNA alone is sufficient to determine the heredity (as will be discussed later), one has often inferred that in the species as low as crystalline viruses the determination of heredity is carried by the ribonucleic acid alone; however, direct evidence is needed before this view can be accepted.

It is interesting to note that in a similar field, that of bacterial viruses, evidence recently has been obtained (23) for the role of nucleic acids as hereditary determinants. This subject will be discussed by Dr. Graham. However, the nucleic acid concerned is not PNA but DNA, and the biological activity of DNA has been already unequivocally proved by the discovery and further study of the transforming phenomenon. The remaining part of the lecture will be devoted to this subject.

The Biological Activity of the Desoxypentose Nucleic Acids—The Transformation Phenomenon

Although the transforming principle was discovered some twenty years ago, the rate of diffusion of this high polymer into scientific circles has been rather slow; this and the recent important progress in the field of the biology

of transforming phenomena make it advisable to discuss first the biology of the phenomenon before we concentrate on the chemistry of it.

Briefly, the transforming phenomenon, discovered in 1928 by Griffith (21), is a permanent change of the hereditary traits of a cell which we may call receptor, into hereditary traits of another cell, which we may call donor, by means of a substance derived from this donor. Once having reached the receptor, the substance usually adds itself permanently to the hereditary apparatus and starts to duplicate, having become part of the gene or the gene itself. The substance has been sometimes called the gene because it has both the main attributes of a gene as usually defined: (1) it self-duplicates and (2) it determines the heredity of the host. Of course to carry out these functions this substance, bearing the name of *Transforming Principle* or *Transforming Agent*, has to meet the proper environment, that is, the interior of the host, but this is also true of the classical "gene"; however, since the actual system performing in the cell may be quite complex, it is perhaps safer to call the transforming principle not the complete gene, but merely the *heredity determinant*. Whatever the name, the transforming principle obviously has properties unknown in any other chemical substance: as a matter of fact, it may be classed together with the simplest known living substances, the crystalline viruses, for the latter also self-duplicate inside the cell and determine their own heredity. Such a claim seems almost exaggerated for a single chemical substance so let's see first whether it is true that the transforming principle is nothing else but a chemical compound. The circumstances in which the phenomenon was first discovered by Griffith (21) didn't make it clear that it is due to chemical substance: what Griffith observed was that, if the receptor cells (rough) were brought in contact with the heat-killed whole donor cells (smooth Type III), these receptor cells acquired a certain hereditary property of the donor, namely that of a production of specific polysaccharide. But what was the *Thing* which passed from the killed donor to the living receptor? In 1932–33 Alloway (6, 7) extracted the killed donor cells and found that the cell-free and partially purified extract had the transforming activity, and it was not until then that the concept of transforming principle as a water soluble substance became feasible. In 1944, Avery, MacLeod, and McCarty (10) purified the extract further and found that it consisted of highly polymerized desoxypentose nucleic acid and had all of its properties. Not only could other substances such as proteins, polysaccharides, or PNA not be detected in it, but of all the enzymes tested, only desoxyribonuclease, that is, the enzyme specific for DNA, could destroy the transforming activity. Thus the transforming principle seems to behave like a well-defined chemical compound, namely DNA; we will have more to say about it later.

What are the biological systems in which the transforming phenomenon has been demonstrated? The original phenomenon was observed on the rough cells of pneumococcus, that is, cells which are unable to produce a specific polysaccharide. Given the DNA fraction derived from the Type III smooth pneumococcus, the rough cells permanently acquired this particular

gene for the production of the Type III polysaccharide, that is the agent in the particular DNA fraction which induced the hereditary property of producing Type III polysaccharide was permanently built into the gene system of the cell. Since this discovery, it was found that the DNA fraction of several types of pneumococcus can be so imposed on certain rough strains. The phenomena is not limited to pneumococcus; it has been also found in *Hemophilus influenzae* (1, 2), and recently also in *Meningococcus* (5). These are the well-authenticated cases; in addition, the phenomenon was also reported in several species of *Enterobacteriaceae* but these experiments have not yet been confirmed.

The phenomenon is not limited to the production of specific polysaccharides. An acquisition of a specific colony morphology in the rough and smooth strains (35), or a specific protein (9), or a specific polyribophosphate (1, 2, 42), or a resistance to antibiotics (25, 26, 4) or change in fermenting properties (36, 8) can be also achieved by transformation. Neither is the phenomenon limited to the acquisition of a previously nonexisting feature. An addition of a new polysaccharide to an existing one, or replacement of the existing one by a new one, has been also achieved (3). This latter phenomenon is of particular interest because here the possible competition of the two DNA's (that is, one determining one type specific substance and another determining another type) for the substrate, that is the receptor, has been suggested by some preliminary experiments. The results also suggest that the law of mass action applies to hereditary determinants. Neither is the phenomenon limited to the achievement of types existing in nature: by the combination of various DNA's in the cell, new "synthetic" types not recognized previously and the corresponding new DNA's, which then breed true, have been obtained (29). The biologists working in this field indeed witness a creation of an entirely new field of genetics, or shall we say chemogenetics, with its specific problems such as whether two specific DNA molecules are situated in the same locus of a chromosome, whether one molecule is a mutant or a combination of others, etc.

The possibility that the molecule which determines the transformation phenomenon may determine the heredity of all living organisms makes a chemical study of the transforming principle urgently needed.

Although the progress in the field of biology of the transforming phenomenon has been quite rapid in recent years, the progress in the field of chemistry, since the discovery of Avery, MacLeod, and McCarty in 1944 (10), was practically none. McCarty found in 1945 (30) that the transforming principle can be reversibly inactivated by ascorbic acid. Hotchkiss (24) studied the absence of proteins in the transforming principle, and that was all. In 1951 we started a chemical study of the transforming phenomenon in *Hemophilus influenzae* and that is what we wish to report here. Because of the magnitude of the problem, the results should be regarded of course merely as the first steps.

We shall begin by describing the typical system in which the transformations are detected.

To 2 cc. of young culture of *H. influenzae* containing approximately one million rough cells and antirough serum to separate by agglutination these original nontransformed cells, one adds 0·1 cc. containing at least 0·0006 γ of a highly polymerized DNA fraction from smooth cells of let us say Type *b*. A 15 min. exposure to DNA is actually sufficient. Afterwards one incubates the system for 18 to 40 hr. and inspects for the appearance of smooth cells of Type *b*. If the DNA has not been tampered with since it was originally isolated, as little as 0·0003 γ DNA per cc. is sufficient to induce uniform transformations. In the above experimental conditions this is still in the best case half a million molecules of DNA per transformed cell and even if we take into consideration the fact that there must be at least as many different DNA molecules as there are genes, 10,000 if you wish, there is still an excess, let us say 50 molecules per transformed cell. Is it because of the small chance of such a large molecule to penetrate or is it because only one molecule in 50 is intact or active? Even if this one is a DNA molecule, does it look exactly like all the others, or is it larger or smaller? This sort of unanswered question may make, of course, some of the chemical interpretations more difficult.

Any chemical study of the DNA having transforming activity must, of course, start with the removal of substances other than DNA. We have reported recently one method of such removal, namely by electrophoresis (41), but it was a rather tedious one. Our new method (40) is based on the observation that when the bacterial cells are lysed with desoxycholate and precipitated by alcohol, the physiological saline extracts from the precipitate the DNA very slowly, but it extracts more rapidly the impurities, that is, the ribonucleic acids and the immunologically active substances. Thus, the precipitate was extracted with small volumes and the first nine extracts were rejected; then it was extracted with large volumes of physiological saline with detergent to deproteinize (31); the last extracts, after having been filtered through celite, were found to contain less than 0·5% of RNA, proteins, or immunologically active substances and that is the best analysis that can be obtained at the present time. Now, on this preparation we have made quantitative studies of resistance to various physical and chemical agents in order to obtain more information on the chemistry of transforming activity. The first agent studied was heat (Fig. 1) (38, 40). As one can see from this figure, both viscosity and activity are remarkably stable when DNA is heated one hour even at 65–75°. Incidentally, when one degrades the DNA, for instance, by drying with alcohol and ether and storing it in a dry state, this stability of viscosity and of activity are irreversibly lost. When heated at higher temperatures, both viscosity and activity, of course, eventually decrease even in good preparations, presumably by breaking weaker bonds, such as perhaps hydrogen bonds. Now the close coincidence of the point of breakdown of the activity of these few molecules of transforming principle,

which we do not see, and the viscosity of the bulk of the DNA preparation, which we do see, suggests again that these two, that is, the transforming principle and DNA are the same substance. It is of interest to note that the viscosities decrease as the logarithms of activity. This may be explained if we realize that a partly broken molecule still contributes to the viscosity, but evidently it cannot be active any more. The exact mathematical analysis would be very complex.

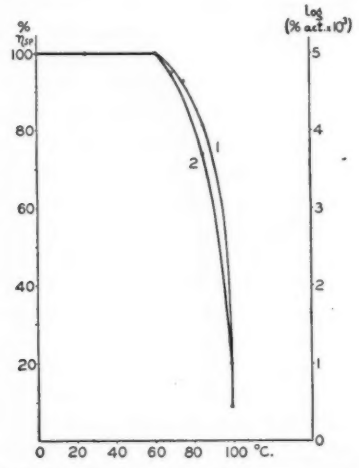


FIG. 1. Stability of DNA of *Hemophilus influenzae* to heat (38, 40). Curve 1, viscosity; curve 2, transforming activity. The ordinates indicate the specific viscosity, measured at 23°, as per cent of maximum viscosity, and the transforming activity as the logarithm of (per cent × 10^3) of maximum activity; the abscissa indicates the temperatures to which the sample was exposed for one hour at pH 7·2 prior to the measurement of the viscosity and activity.

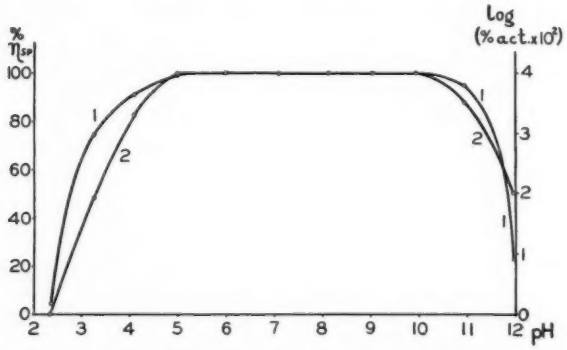


FIG. 2. Stability of DNA of *Hemophilus influenzae* to pH changes (38, 40). Curve 1, viscosity; curve 2, transforming activity. The ordinates indicate the specific viscosity as per cent of maximum viscosity, and the transforming activity as the logarithm of (per cent × 10^2) of maximum activity; the abscissa indicates the pH to which the sample was exposed for two hours at 23°.

The next study was the action of the change of the hydrogen ion concentration (Fig. 2). The region of stability of DNA of *H. influenzae* which can live in the blood at pH 7·4 is remarkably symmetrical with respect to pH 7·4.

Now it is to be noted that the points at which the transforming activity breaks down on the acid and alkaline sides coincide very well with the points of breakdown of the viscosity of the DNA preparation (38, 40), indicating again that the transforming principle is the DNA. One of the best proofs that the transforming principle is DNA was the study of Avery, MacLeod, and McCarty (10) in which, of many enzymes tested, only the desoxyribonuclease in minute amounts was able to destroy the activity completely. This finding was corroborated by Hotchkiss (24) and by Alexander and Leidy (1, 2). In order to obtain more information on this subject, we have tested quantitatively the effect of crystalline pancreatic desoxyribonuclease on viscosity and on transforming activity in the concentrations of the enzyme as low as 0·0007 γ /cc. (Fig. 3) (38, 40). As we can see from this figure even this small amount of the enzyme specific for DNA is sufficient to inactivate the transforming principle. However, the picture of the loss of viscosity is entirely different from the ones observed before in the case of heat and pH changes. Here, a very small (if any) loss of viscosity of the bulk of the preparation corresponds to the complete loss of activity. Evidently the enzyme first breaks some vital bonds which does not decrease the size of the molecule but completely denatures it.

Another study of denaturation was made by changing the ionic strength of the solution, starting with the 0·14 *M* sodium chloride solution and going down to distilled water (Fig. 4) (38, 40). When the solution is restored to the original 0·14 molar salt concentration, one can see that the viscosity

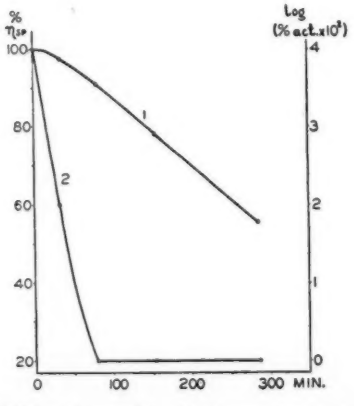


FIG. 3. Stability of DNA of *Hemophilus influenzae* to crystalline pancreatic desoxyribonuclease (38, 40). Curve 1, viscosity; curve 2, transforming activity. The ordinates as per Fig. 2: the abscissa indicates time of incubation with 0·0007 γ/cc. desoxyribonuclease at 29·6°.

(Curve 1) has not changed but the activity (Curve 2) has completely disappeared. This indicates that the asymmetry of the molecule might not have suffered, yet the molecule has been completely denatured. The latter may be due to breakage of some vital bonds (H-bonds?) during the stretching (uncoiling) of the DNA molecules caused by repulsion of the negative charges in the solution of low ionic strength.

An entirely different type of reaction was found in the study of formylation. The formaldehyde has been used for research in inactivation of antibodies, enzymes, and viruses. It forms, of course, compounds with free amino groups but, if the reaction has not proceeded for too long a time, the bond is not strong and can be split by acid or even by dialysis, with partial reactivation of the protein.

To our knowledge the reaction between intact desoxyribonucleic acid and formaldehyde has never been studied. One could expect that the formaldehyde will react with the free primary amino groups of adenine, guanine, and cytosine and this indeed seems to be the case. When exposed to 4 *M* formaldehyde for two and one-half hours, the formaldehyde was bound to the extent of more than 10% of the primary amino groups of DNA; as in proteins, the bond was rather labile and formaldehyde could be removed (and measured) by mild treatment with acids and by prolonged dialysis; this latter procedure, however, fails to remove the last 10% of the originally bound formaldehyde. Now if one follows the loss of viscosity and the loss of activity of transforming principle in such an exposure to formaldehyde, one arrives at the following picture (Fig. 5) (38, 40). The exposure for the time shorter than two and one-half hours at room temperature results in practically no decrease of viscosity or activity. At two and one-half hours and after, the formaldehyde

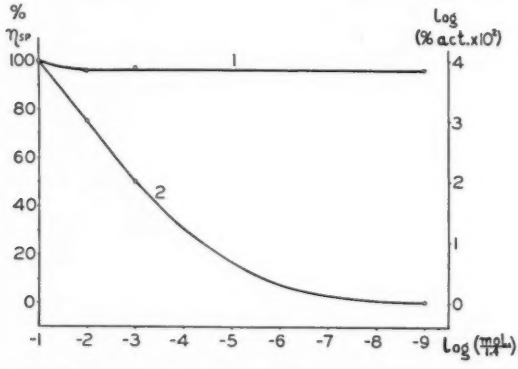


FIG. 4. Stability of DNA of *Hemophilus influenzae* to ionic strength changes (38, 40). Curve 1, viscosity; curve 2, transforming activity. The ordinates indicate the specific viscosities (all measured in 0·14 *M* sodium chloride solution at 23°) as per cent of maximum viscosity, and the transforming activity as the logarithm of (per cent × 10^2) of maximum activity; the abscissa indicates the logarithm of (molarity/1·4) of aqueous sodium chloride solution to which the sample was exposed for 24 hr. at 8° prior to testing.

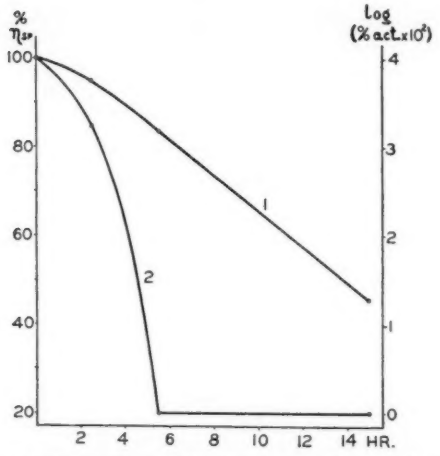


FIG. 5. Stability of DNA of *Haemophilus influenzae* to formaldehyde (38, 40). Curve 1, viscosity; curve 2, transforming activity. The ordinates as per Fig 2; the abscissa indicates time of exposure to 4 *M* formaldehyde at 23° and pH 7·2.

probably competes efficiently for the primary amino group with some bonds (probably H-bonds) holding the molecule in its original shape, with the result that some of these bonds are broken and the viscosity decreases; such breaking results in an extremely rapid inactivation of the transforming principle. It appears therefore that if the molecule of the transforming principle behaves like a typical DNA molecule, the covering of some of the free amino groups still allows it to act as an hereditary determinant provided that the reaction does not result in the decrease of the asymmetry of the DNA molecule.

All these studies are, of course, still far from concluded. It seems, however, that even the preliminary results presented here may be of some use in understanding the denaturation of DNA in general, and of the molecule which determines the heredity in particular. It is possible that this kind of study will finally demonstrate whether some of the chemically reactive groups are more vital for the determination of heredity than others.

References

1. ALEXANDER, H. E. and LEIDY, G. Proc. Soc. Exptl. Biol. Med. 73 : 485. 1950.
2. ALEXANDER, H. E. and LEIDY, G. J. Exptl. Med. 93 : 345. 1951.
3. ALEXANDER, H. E. and LEIDY, G. Proc. Soc. Exptl. Biol. Med. 78 : 625. 1951.
4. ALEXANDER, H. E. and LEIDY, G. J. Exptl. Med. In press.
5. ALEXANDER, H. E. and REDMAN, W. J. Exptl. Med. In press.
6. ALLOWAY, J. L. J. Exptl. Med. 55 : 91. 1932.
7. ALLOWAY, J. L. J. Exptl. Med. 57 : 265. 1933.
8. AUSTRIAN, R. Personal communication.
9. AUSTRIAN, R. and MACLEOD, C. M. J. Exptl. Med. 89 : 451. 1949.
10. AVERY, O. T., MACLEOD, C. M., and MCCARTY, M. J. Exptl. Med. 79 : 137. 1944.
11. BERNHEIMER, A. W. Biochem. J. 53 : 53. 1953.
12. BERNHEIMER, A. W. and RUFFIER, N. K. J. Exptl. Med. 93 : 399. 1951.
13. CECIL, R. and OGSTEN, A. G. J. Chem. Soc. 1382, 1948.
14. CHARGAFF, E. Federation Proc. 10 : 654. 1951.

15. CREETH, J. M., JORDAN, D. O., and GULLAND, J. M. J. Chem. Soc. 1406. 1949.
16. ELSON, D. and CHARGAFF, E. Federation Proc. 10 : 180. 1951.
17. ELSON, D. and CHARGAFF, E. Experientia, 8 : 143. 1952.
18. FAIRLEY, J. L., SEAGRAM, M. L., and LORING, H. S. Federation Proc. 9 : 169. 1950.
19. FRAENKEL-CONRAT, M. L. and DUCAY, E. D. Biochem. J. 49 : XXXIX. 1951.
20. GOLDSTEIN, G. and STERN, K. G. J. Polymer Sci. 5 : 687. 1950.
21. GRIFFITH, F. J. Hyg. 27 : 113. 1928.
22. HENRY, H. and STACEY, M. Proc. Roy. Soc. (London), B, 133 : 391. 1946.
23. HERSHEY, A. D. and CHASE, M. J. Gen. Physiol. 36 : 39. 1952.
24. HOTCHKISS, R. D. Colloques int. Centre Natl. Recherche Sci., Unités biol. douées contin. génét. 8 : 57. 1948.
25. HOTCHKISS, R. D. Cold Spring Harbor Symposia Quant. Biol. 16 : 457. 1951.
26. HOTCHKISS, R. D. II-e Congrès Int. de Biochimie. Symposium sur le mode d'action des antibiotiques (Paris). 1952, p. 40.
27. JEENER, R. and BRACHET, J. Enzymologia, 11 : 222. 1943–45.
28. KAUFMAN, B. P., MCDONALD, M., and GRAY, H. Nature, 162 : 814. 1948.
29. LEIDY, G., HAHN, E., and ALEXANDER, H. E. J. Exptl. Med. In press.
30. MCCARTY, M. J. Exptl. Med. 81 : 501. 1945.
31. MARKO, A. M. and BUTLER, G. C. J. Biol. Chem. 190 : 165. 1951.
32. MARSHAK, A. J. Biol. Chem. 189 : 607. 1951.
33. NEURATH, H., GREENSTEIN, J. P., PUTNAM, F. W., and ERICKSON, J. O. Chem. Revs. 34 : 157. 1944.
34. POLLISTER, A. W., and LEUCHTENBERGER, C. Nature, 163 : 360. 1949.
35. TAYLOR, H. E. J. Exptl. Med. 89 : 399. 1949.
36. TAYLOR, H. E. Personal communication.
37. VOLKIN, E. and CARTER, C. E. J. Am. Chem. Soc. 73 : 1516. 1951.
38. ZAMENHOF, S. *In* Phosphorus metabolism. Vol. 2. *Edited by* McElroy, W. D. and Glass, H. B. John Hopkins Press, Baltimore, Md. 1952. p. 301.
39. ZAMENHOF, S. and CHARGAFF, E. J. Biol. Chem. 180 : 727. 1949.
40. ZAMENHOF, S., LEIDY, G., and ALEXANDER, H. E. II-e Congrès International de Biochimie, Résumés des Communications (Paris). 1952. p. 208.
41. ZAMENHOF, S., LEIDY, G., ALEXANDER, H. E., FITZGERALD, P. L., and CHARGAFF, E. Arch. Biochem. 40 : 50. 1952.
42. ZAMENHOF, S., LEIDY, G., FITZGERALD, P. L., ALEXANDER, H. E., and CHARGAFF, E. Federation Proc. 11 : 315. 1952; J. Biol. Chem. In press.
43. ZAMENHOF, S., SHETTLES, L. B., and CHARGAFF, E. Nature, 165 : 756. 1950.

DISCUSSION

Q.—**Dr. K. W. McKerns** *(Canada Packers, Limited, Toronto)*: Does the DNA of *Hemophilus influenzae* differ from that of other viruses?

A.—**Dr. Zamenhof:** Yes. The DNA of *Hemophilus influenzae* exhibits a ratio of adenine to guanine of 1·75 and of thymine to cytosine of 1·54. These ratios are characteristic for this species and differ from those of the DNA's of other organisms.

Q.—**Dr. E. G. Young:** What changes characterize the denaturation of nucleic acids?

A.—**Dr. Zamenhof:** The most characteristic change is the loss of biological activity (transforming activity in DNA) but thus far only a few systems are amenable to this test. In the DNA the denaturation is often (but not invariably) accompanied by a decrease in viscosity, stability to heat, and changes in the characteristics of the titration curve.

Q.—**Dr. R. K. Stewart** (*Dalhousie University*): What is polyribophosphate?

A.—**Dr. Zamenhof:** Polyribophosphate is a newly found substance in the capsule of type-*b Hemophilus influenzae*. Its structure is similar to that of ribonucleic acid except that it contains no purines or pyrimidines (Zamenhof *et al.* Fed. Proc. 11 : 315. 1952).

THE DESOXYRIBONUCLEIC ACID OF INTERPHASE AND DIVIDING NUCLEI[1]

By C. E. Stevens, R. Daoust[2], and C. P. Leblond

Abstract

Classical genetics has associated the rigid transmission of hereditary characters with the constancy of the chromosome pattern. Modern work has shown that the chromosomes contain the substance desoxyribonucleic acid (DNA), which is believed to be the carrier of genetic potential. In general, the amount of this material present per cell is constant in all organs and tissues in a given species. Furthermore, isotopic work with a DNA precursor, phosphorus, showed that it does not enter the DNA of tissues in which the cells do not divide—a result interpreted as supporting the classical concept of the stability of genetic material. It was known, on the other hand, that phosphorus is incorporated into the DNA of tissues containing cells undergoing division—a result interpreted as being due to the synthesis of DNA associated with cell division. Evidence is presented to indicate that such synthesis does occur, but is far more extensive than was hitherto suspected, since it involves the complete replacement of all the DNA present before initiation of the cell division. How the cell can safeguard the stability of the genetic material in the course of this total DNA renewal can only be surmised at this time.

When the division of cells by mitosis was discovered (1879), it was soon realized that this process resulted in an equal distribution of the chromosomes between the two daughter cells. Toward the turn of the century Weissman suggested that the nucleus is responsible for the transmission of hereditary characters. It then became apparent that the equal partition of chromosomes also implied an equal distribution of genetic material between the two daughter cells produced by mitosis.

Meanwhile, Miescher isolated a "nucleic acid" which he claimed was the main constituent of the nucleus. Biochemical studies showed that this acid, DNA, contained phosphorus and a desoxypentose sugar related to ribose. Histochemical work showed that the chromosomes were the loci of the DNA. This fact, and many other lines of evidence obtained from work on the induction of mutations by agents which affect DNA, as well as the studies on the viruses and on the transforming principle in bacteria, suggested that DNA is largely responsible for the maintenance of heredity. This conclusion has greatly stimulated the study of this chromosome component.

In this paper, an attempt is made to develop some of the current ideas on the behavior of DNA in the interphase and dividing nucleus. A brief description of the location of DNA in cells is followed by a demonstration of the constancy of the amount of DNA in nondividing nuclei. The time at which DNA synthesis occurs in relation to mitosis is then discussed. Finally, the evidence suggesting that this synthesis is associated with a complete renewal of DNA is presented.

[1] *Manuscript received February 2, 1953.*

Contribution from Department of Anatomy, McGill University, and Montreal Cancer Institute, Notre-Dame Hospital, Montreal, Quebec. Aided by a grant from the National Cancer Institute of Canada.

[2] *Fellow of the Damon Runyon Memorial Fund for Cancer Research.*

Location of DNA in the Cells

The localization of DNA has been greatly facilitated by use of the Feulgen nuclear reaction. The specificity of this technique has been shown in several ways (51). Thus, films of DNA can be stained by the Feulgen reaction, while after treatment with the enzyme, desoxyribonuclease, such staining fails to occur. The numerous investigations carried out by means of the Feulgen reaction led to an outstanding conclusion, namely, that all the DNA present in the cell is concentrated in the chromosomal material of the nucleus.

In general examination of a nondividing or interphase nucleus stained with Feulgen shows scattered granules or particles, the size and number of which differs in cells of various types. There is also a moderate staining of the nuclear membrane. Occasionally, filaments seem to be present. These observations have been of little help in the understanding of the nuclear structure and function.

More significant results were obtained by examining the nuclei of certain eggs. Thus, Duryee (16) showed that at certain stages of development, the live interphasic nucleus of the frog's egg displays threadlike structures (Fig. 1) which, in fixed preparations, give the Feulgen reaction (15). These structures —or chromosomes— are composed of a fine filament, which supports granules of various sizes scattered along its length (Fig. 2). The filament may be referred to as a "chromonema" and the granules, as "heterochromatin". (In addition, the chromosomes elaborate and release Feulgen negative material which is described as "loops" and was postulated to be the instrument by which the chromosomes transfer their influence to the cell (15, 16).) Finally, the fine chromonemata and associated heterochromatin appear to be enclosed in a vesicular sheath which does not stain with Feulgen.

The chromosomes may also be readily seen in living salivary gland cells of *Drosophila*. Here, each chromosome is believed to consist of a number of chromonemata lying side by side. Each chromonema carries heterochromatin granules of various sizes. The corresponding granules of adjacent chromonemata are arranged across the width of the chromosomes in the form of bands. Ris and Crouse (46) believe that the heterochromatin granules may be nothing more than closely packed coils of the chromonema. More generally, however, it is believed that each granule corresponds to an accumulation of extraneous DNA at a given point on the chromonema. In any case, the heterochromatin granules represent the gene loci of modern genetics. Finally, the work of d'Angelo (12) indicated that the chromonemata of resting nuclei are also enclosed in a vesicular sheath, which is visible only if outlined by fine carbon particles injected into the nucleus. Thus, a chromosome would be composed of chromonema, heterochromatin granules, and a vesicular sheath.

In the two examples just cited (frog's egg, salivary gland) the chromosomes were visible in both living and Feulgen stained interphase nuclei. In most cells, however, they are not recognizable on similar microscopic examination. This is probably due to the fact that the thickness of the chromonemata is less

PLATE I

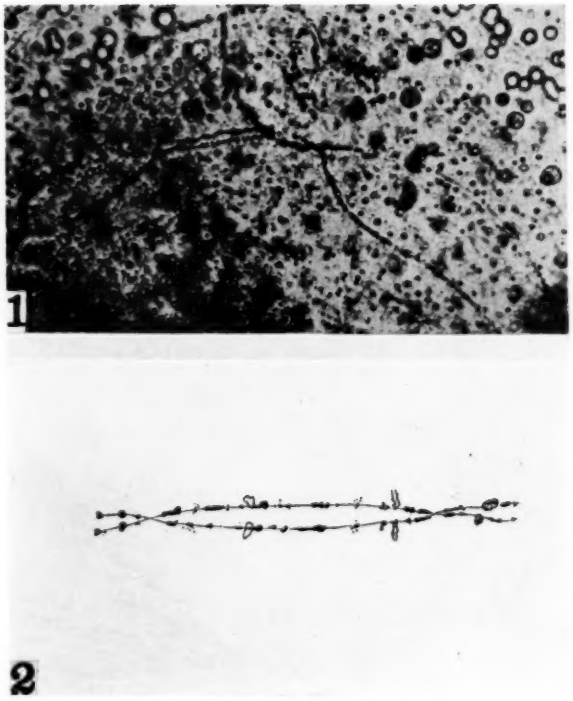
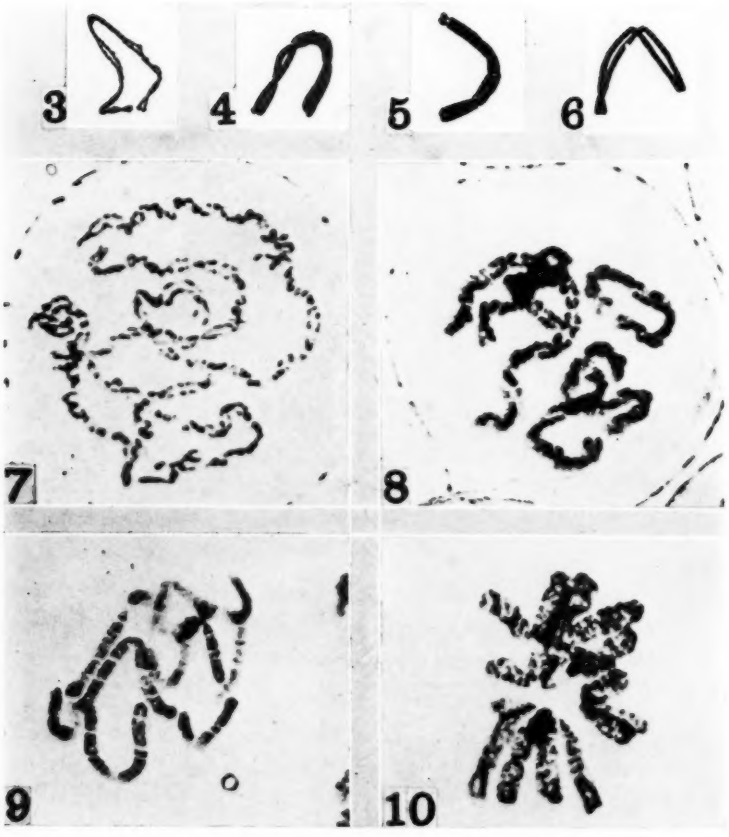


FIG. 1. Filaments visible in the nucleus of the frog's egg and identified as chromosomes.

FIG. 2. Camera lucida sketch of same. Note the filament itself (chromonema), the granules, and the loops.
(From Duryee (16).)



FIGS. 3-6. Chromosomes of dividing cell of onion root. The midprophase shows the two chromatid filaments (Fig. 3) which at metaphase appear thicker (Figs. 4 and 5), and, at the end of metaphase, separate (Fig. 6).
(After L. Vanderlyn (54).)

FIGS. 7–10. Showing some detail in the structure of the chromosomes of dividing microspores of *Tradescantia grandiflorum*. The early prophase shows the two coiled chromatid filaments (Fig. 7), the coils of which become tighter as the structure gradually shortens at late prophase (Fig. 8), early anaphase (Fig. 9), and late anaphase (Fig. 10). How the packing of the coils of the chromonema can make the chromosome appear as a short rod may be inferred from Fig. 9.
(After A H. Sparrow (48).)

than the resolution of the microscope. In fact, electron microscope preparations of the nuclei of rat spermatocytes showed the presence of paired threads which seemed to originate from large, irregular masses in contact with the nuclear membrane (57; Plates 35 and 36). We interpret the threads as chromonemata and the irregular masses as heterochromatin (which in this case, adheres to the nuclear membrane). Since the threads have a diameter of only 0.04μ (57), they would presumably escape investigation with ordinary microscopes. Threadlike structures can, however, be seen with the light microscope in certain cells, especially following their isolation from interphase nuclei by mechanical disruption and differential centrifugation. Thus, Claude and Potter (11) used leukemic cell nuclei, which in Feulgen-stained sections displayed a network of fine reactive filaments. Grinding and centrifuging these nuclei made it possible to isolate threads which these authors identified as chromosomes. After chloroform extraction and Feulgen staining, the threads showed beadlike formations (11) which may consist of heterochromatin. Mirsky and Ris extracted similar filamentous structures from the nuclei of liver, kidney, thymus, and pancreas. Furthermore, since 90% of this material behaved like DNA protein, they concluded that the threads were, undoubtedly chromosomes (35, 36).

The differences observed in the various types of interphase nuclei appear to be due only to variations in the mode of packing of the chromonema threads. Such variations may be ascribed on the one hand to the size of the nucleus. Thus, when the nucleus is large (as in certain nerve cells), the chromonemata would be separated from one another, and the Feulgen-stained nucleus would appear as a transparent body. When the nucleus is small (as in lymphocytes), the chromonemata would be tightly packed and the nuclei would accordingly stain more intensely. A second factor may be that the heterochromatin granules associated with the chromonemata tend to adhere to each other (25, 53) in certain cell types. (This may be the case when, in the testis, the finely granular nuclei of Type A spermatogonia give rise to the coarsely granular nuclei of Type B spermatogonia.) Finally, the observation that the Feulgen stain often outlines the nuclear membrane (and occasionally also that of the nucleolus) might be explained by the frequent adhesion of heterochromatin granules to these membranes (25, 53).

The variegated picture presented by Feulgen-stained nuclei in various types of cells may be contrasted with the similarity of the Feulgen-stained chromosomes in any dividing cell examined in a given species. Their number as well as their form is indeed characteristic of the plant or animal species; and their equal division proceeds so as to ensure that the two daughter nuclei contain exactly the same chromosomes as the parent, thus maintaining the constancy of the pattern.

At the beginning of the prophase period, chromosomes appear to be composed of two identical chromatids, each of which consists of at least one chromonema with associated heterochromatin (Figs. 3 and 7). The prophase is characterized by a shortening and a coiling of the chromonemata (Figs. 3 to

10; see also Ref. 26*a*). In most animal cells, neither the chromonema coils, nor the individual heterochromatin granules can be distinguished at metaphase, since each chromosome condenses to a short, solid rod. These chromosomes also are included in a matrix (which may be a condensation of the vesicular sheath described above). There is much controversy regarding the fate of the chromosome components. Hughes expresses the widespread opinion that, as the vesicular sheath of each chromosome swells in the course of anaphase and telophase, most of the DNA is dissolved out of the chromonema and heterochromatin granules and is dispersed in the vesicular fluid, while the rest of the DNA remains associated with the structures throughout the interphase (25). A radically different opinion is that of Ris, according to whom chromonemata may coil to a considerable extent and heterochromatin granules would be merely due to the close juxtaposition of such coils. The packing of the chromonema coils would reach a maximum at or around metaphase—resulting in the solid, rodlike chromosome seen at that time (46). There may well be room for an intermediate opinion according to which chromonemata and heterochromatin are two distinct structural entities, both of which would persist throughout all phases of the mitotic cycle. As evidence for this hypothesis no dissolved DNA could be found in the nuclear sap of *Xenopus* ovocytes (10). Therefore, in these and possibly in other nuclei as well, there is no departure of DNA from the chromosomes at the end of mitosis. The shortening of chromonemata at prophase (26*a*, 48) would result in a close packing of the heterochromatin as well as of the chromonemata themselves, and thus cause the rodlike appearance of metaphase chromosomes. Conversely, the lengthening of the chromonemata at telophase would make them too fine to be seen in resting nuclei.

In conclusion, it is now clear that resting as well as dividing cells contain chromosomes. Indeed, the three main components of chromosomes, namely, chromonema, heterochromatin, and vesicular sheath, persist throughout the life of the cell. It is suggested that DNA is associated with the first two elements only.

The essential function of mitosis seems to be to duplicate the chromosomes, particularly the DNA containing chromonemata and heterochromatin, thereby providing each daughter cell with the number of chromosome pairs characteristic of the species involved.

Amount of DNA per Nucleus

The evidence presented indicates that DNA forms an essential part of chromosomes. With a few exceptions to be mentioned below, all cells of the body in a given species contain a constant number of well-defined chromosome pairs. To determine whether the cells also contain a constant amount of DNA, Boivin and the Vendrelys (9) estimated the DNA content, as well as the number of nuclei present, in homogenized tissues. From these data,

TABLE I

AMOUNT OF DNA PER NUCLEUS IN VARIOUS TISSUES OF THE CALF AND OX, AS ESTIMATED CHEMICALLY IN TISSUE HOMOGENATES

(Micrograms $\times 10^{-6}$)

	Data of Vendrely and Vendrely (55)	Data of Mirsky and Ris (37)
Thymus	6·4	7·15
Liver	6·4	6·22 (calf) 8·4 (adult)
Kidney	5·9	6·25 (calf) 6·81 (adult)
Pancreas	6·9	
Sperm	3·3	2·82

the amount of DNA per nucleus may be readily calculated. The result gives the mean value for all the nuclei in an organ, including those of connective tissue and blood vessels (Table I).

The quantity of DNA per nucleus may also be estimated by the photometric method, a technique used with success by Swift (52), Pasteels and Lison (32, 33, 39, 40, 41), and others. Histological sections prepared by methods preserving nucleic acids were stained by Feulgen's nucleal technique. The magnified image of a single nucleus was projected onto the window of a photoelectric cell. The light absorbed by the dye in the nucleus was measured, the amount of dye being proportional to the amount of DNA (39). The results were expressed in arbitrary units (Table II).

The *chemical analyses* of the tissues of the calf and the ox by Vendrely and Vendrely (55, 56) and Mirsky and Ris (37) concur in showing that the amount of DNA per nucleus is remarkably uniform in all somatic cells (Table I). The constancy of the amount of DNA measured may be related to the fact that most somatic cells contain the same set of chromosome pairs (diploid nuclei). On the other hand, the sex cells, sperm and ova, contain only a set of single chromosomes (haploid nuclei). Accordingly, sperm cells of bulls contain half the amount of DNA of the somatic cells (Table I). This result was confirmed in several species (14).[1]

By *photometric measurements* of the tissues of mice, Swift (52) and Lison and Pasteels (32, 39, 40) also found that the average amount of DNA per nucleus was the same in all diploid somatic cells (Table II, "Class I"). Some nuclei (called "Class II" or tetraploid) contained twice as much, and a few nuclei

[1] *From the amount of DNA in the human spermatozoon, namely, 3·4 to 4·4 $\times 10^{-12}$ gm. (13) and assuming that the molecular weight of DNA varies from 1·0 to 1·3 $\times 10^6$, the number of molecules per nucleus can be calculated (6·06 $\times 10^{23}$ being taken as Avogadro number) to be approximately 2 $\times 10^6$.*

Since in man there appears to be at least 10^6 genes (49), there would thus be no more than 20 molecules per gene.

TABLE II

AMOUNT OF DNA PER NUCLEUS IN VARIOUS TISSUES OF THE MOUSE, AS ESTIMATED PHOTOMETRICALLY IN SECTIONS

(Arbitrary units)

	Data of Swift (52)		
	Class I	Class II	Class III
Liver	3·34	6·77	13·2
Pancreas	3·10	6·36	12·4
Thymus	3·28	6·17	
Lymphocytes	3·20	6·00	
Kidney tubule	3·14		
Small intestinal epithelium	2·97		
Spleen	3·12		
Neurones (spinal cord)	3·14		
Testis:			
Interstitial cells	3·05		
Sertoli cells	3·00	6·40	
Spermatids 1·68			

(called "Class III") contained four times as much DNA as the Class I nuclei. This occasional replication of the amount of DNA is in agreement with evidence obtained by cytogeneticists for replication of the chromosome complement, or "polyploidy", observed in certain somatic cells, particularly those of liver and pancreas.[2]

In the testis, a Class I amount of DNA was usually observed in interstitial and Sertoli cells; and these, therefore, are diploid cells (Table II). Spermatids contained half this amount, as expected from the fact that spermatids are the precursors of spermatozoa and, like them, are haploid. Photometric measurements of the amount of DNA in the cells of the mouse ovary also showed that the mature egg cell contains half the amount of DNA present in most somatic cells (5).

In conclusion, the interphase nuclei of most somatic cells of a given species are diploid and contain equal amounts of DNA. A few, however, are polyploid, i.e., contain two or more times the diploid number of chromosomes, and their DNA content is increased accordingly. Conversely, the haploid male and female germ cells contain half the usual, or somatic, amount of DNA.

Formation of Additional DNA in Relation to Mitosis

When the cell divides, the DNA is distributed between the two daughter nuclei. Both must eventually contain the same amount as the parent nucleus. Therefore, an amount of DNA equal to that in the parent nucleus must be synthesized at some stage in the process of cell division.

[2] *Recent chemical analyses have also revealed a higher DNA content of the average nucleus in organs showing many polyploid nuclei, such as the liver of rats and mice (14, 20).*

In the past, examination of histological preparations had suggested that the DNA of the parent nucleus increased during prophase, that is, at the first visible stage of division. However, Price and Laird (44), using chemical estimations in partially hepatectomized rats, observed that prior to the appearance of mitotic figures, the average DNA content of the liver nuclei had increased from 10 to 18.3 $\times$ 10^{-12} gm. Thus, DNA synthesis had preceded morphological cell division. Independently, Swift (52) came to the same conclusion, since photometric measurements of individual nuclei revealed that their DNA content at the earliest stages of prophase was twice that of telophase or early interphase nuclei (see also (47)).

Thus, in general, duplication of DNA takes place in the parent cell prior to division, and well before any visible signs indicate that the cell is about to divide.

The DNA content of interphase nuclei will, therefore, be increased in cells preparing for division. Thus, the Class II nuclei of Swift do not necessarily consist of truly tetraploid nuclei, since some may be nuclei that have doubled their DNA content prior to mitosis, especially in organs such as the thymus where mitoses are common. (It is, however, not known whether the duplication of the DNA content is associated with the doubling of the number or size of chromonemata and associated heterochromatin.)

The amount of DNA present in the nucleus permits a subdivision of the interphase into the following three stages:

(*a*) *Stable stage*, which follows telophase and during which the nucleus maintains a constant, basal amount of DNA (characteristic of the degree of ploidy of the cell under consideration).

(*b*) *Duplication stage*, during which the DNA content of the nucleus gradually increases until it reaches twice the basal amount.

(*c*) *Postduplication stage*, during which the amount of DNA present in the nucleus is twice the amount present during the stable stage. The initiation of the visible events of cell division (prophase) marks the end of this stage.

The duration of the *stable stage* seems to vary inversely with the rate at which cells divide. When mitoses are absent (neurones) or rare (kidney), all nuclei will usually be reported as Class I (Table II). Such nuclei remain indefinitely at the stable stage. In contrast, the stable stage is very short in the cells of tissues having a high mitotic activity. Thus, most interphase nuclei in the root tips of *Zea mais* have twice the DNA content of telophase nuclei (52), and therefore, the stable stage must have been of short duration and soon followed by the other stages. In segmenting eggs of *Paracentrotus lividus* (33) and in the epithelium of the crypts of Lieberkühn in the rat intestine (40), mitoses succeed each other so rapidly that the duplication stage coincides with the telophase of the previous mitosis, and hence, there is no stable stage at all.

[3] *From the comparison of the isotope content of DNA with that of the incubation medium, values of 3% and 4% were obtained for the daily formation of DNA purines and phosphorus moieties respectively.*

The duration of the *duplication stage* is rather short. In the last two examples, it is less than that of telophase, since an advanced telophase nucleus contains twice the DNA content of an early telophase nucleus. Thus, the DNA synthesis must occur rather rapidly—probably in a matter of minutes rather than hours. The brevity of the duplication stage is also indicated by the fact that, in general, the number of nuclei with a DNA content intermediate between those of the stable and postduplication stages is low.

The duration of the *postduplication stage* showed relatively little variation. Using a method to be described below, it was estimated that the synthesis of DNA in the cells of the bean root *(Vicia faba)* was completed about six hours before mitosis (23). In the crypts of Lieberkühn of the rat intestine, the occurrence of the duplication stage at telophase (40) indicates that the postduplication stage coincides with the interphase. Thus, the duration of the postduplication stage equals that of the intermitotic time, which, from published (29) and unpublished data, can be calculated to be approximately 11 hr. in this tissue.

In tissues in which abundant mitoses can be produced experimentally, the combined duration of duplication and postduplication stages may be estimated from the time elapsing between the application of the stimulus and the appearance of mitosis. Thus, injection of prolactin into a pigeon will induce the appearance of mitosis within nine hours (28). Following partial hepatectomy, DNA synthesis starts at once, but mitoses do not appear in abundance until 24 hr. after the operation (27, 44). Thus, all the data available to date indicate that the duration of the postduplication stage is between 6 and 24 hr.

In conclusion, the *mitotic cycle* may be considered to be initiated by the rather short "duplication stage". The 6–24 hr. postduplication stage, and later the four visible phases of division—prophase, metaphase, anaphase, and telophase—follow. The mitotic cycle is concluded by the stable stage, during which the cell presumably exercises its specialized functions.

Incorporation of Radioisotopes into DNA at the Various Stages of the Mitotic Cycle

The synthesis of DNA during the "duplication stage" makes it possible to supply the same basal amount of DNA to all cells arising from mitosis, thus fulfilling the classical genetic postulate of stability of the hereditary units. To establish definitely whether the DNA molecules, once formed, are stable, or whether there is some metabolic renewal of their components, radioisotopes were used.

(a) Incorporation of Radiophosphorus into DNA

Some of the earliest work along these lines was on the metabolism of phosphorus of nucleic acids, using the P^{32} isotope of phosphorus. These biochemical studies established that the entry of P^{32} into DNA was negligible in tissues without dividing cells (38) and was roughly proportional to the incidence of mitoses in other tissues (Table III, and Refs. 22 and 26).

TABLE III

RATE OF INCORPORATION OF PHOSPHORUS INTO DNA
(RATIO OF THE SPECIFIC ACTIVITY OF DNA P TO THAT OF INORGANIC P (× 100) IN VARIOUS ORGANS OF THE RAT TWO HOURS AFTER P^{32} ADMINISTRATION)

	Data of Ahlström, Euler, and Hevesy (4)
Intestinal mucosa	4·80
Spleen	2·50
Kidney	0·16
Liver	0·14

These observations were confirmed by the radioautographic technique. Subsequent to injection of P^{32}, sections of animal tissues may be treated so as to retain DNA as the only phosphorus-containing compound. By the "coating" autographic technique, such tissue sections can be used to localize labeled DNA. Hence, in various tissues, the areas of cells involved in mitotic division were observed to affect the emulsion, while areas of differentiated cells failed to show any reaction. Furthermore, the labeling with P^{32} of the cells arising from mitosis made it possible to trace their displacement from their site of origin (30). It may be concluded from these biochemical and radioautographic studies that the incorporation of P^{32} into DNA is closely associated with the mitotic process.

It was important to examine the problem in detail, using cells large enough for the radioautography of individual nuclei. Such nuclear autographs were obtained by Howard and Pelc (23, 24, 43), using roots of *Vicia faba*. Following immersion of these roots in a solution of radiophosphate they observed no autographs in the areas without mitotic activity, while in areas with abundant mitoses, the autographs at first overlaid interphase nuclei and, 6–24 hr. later, prophases and other stages of division. They concluded that phosphorus enters nuclei about six hours before prophase, presumably at the time when DNA is duplicated.

In conclusion, formation of DNA phosphorus takes place exclusively in cells preparing for mitosis, that is, during the duplication stage of the interphase. During the other stages of the mitotic cycle, the DNA molecules remain stable, at least as far as the phosphorus component is concerned. Stability of the phosphorus moiety probably implies stability of the backbone of the DNA molecule in which the phosphate groups alternate with the sugar residues (6).

(b) Incorporation of Other Isotopes into DNA

While the backbone of the DNA molecule remains intact, some of the purine and pyrimidine bases (protruding from the backbone like the teeth of a comb) might be replaced with or without affecting the over-all nucleotide sequence.

To investigate this possibility, the uptake of various labeled substances by the purines and pyrimidines of DNA has been investigated. Abrams, for instance, has reported that labeled adenine and guanine are incorporated into the DNA, of bone marrow slices at rates similar to that at which P^{32} is incorporated (2, 3)—a fact which suggests that the entry of adenine and guanine also takes place in association with mitosis.

Some information on the relation between mitotic activity and the rate of entry of various labeled substances into DNA can be obtained from comparison of the radioactivity of DNA in various tissues. Recent data of this type, obtained with labeled formate by Goldthwait and reported by Bendich (7), have suggested that the rates of incorporation of labeled formate into the bases of DNA in various organs of the rat parallel the mitotic activities of these organs (Table IV).

TABLE IV

RATE OF INCORPORATION OF C^{14} FORMATE INTO DNA
(SPECIFIC ACTIVITY OF BASES IN VARIOUS ORGANS OF THE RAT AFTER C^{14} FORMATE ADMINISTRATION, IN COUNTS PER MINUTE PER MICROMOLE)

	Unpublished data of Goldthwait, reported by Bendich (7)		
	Adenine	Guanine	Thymine
Small intestine	213	354	390
Large intestine plus stomach	89	103	142
Spleen	39	44	41
Testes	8	1	18
Kidney	4	8	12
Liver	7	7	8

In the literature far more emphasis has been placed on a type of work in which the uptake of various labeled compounds by DNA is assessed by measuring the content of given isotopes in DNA relative to the content in RNA. Such a method of estimation may be misleading, since various labeled compounds may show differences in regard to *(a)* their relative availability for DNA and RNA (i.e., their relative uptake by immediate precursors of DNA and RNA) and *(b)* their qualitative labeling of DNA and RNA (some precursors may be incorporated selectively into some DNA or RNA moieties (42)). Accordingly, it may be necessary to re-examine completely the conclusions drawn from DNA:RNA ratios obtained with various precursors and, particularly, the widely accepted hypothesis that the DNA synthesis has two pathways: one involving the incorporation of phosphorus

and preformed purines during the mitotic process[4] and one involving a partial renewal of the nitrogenous bases from small molecule precursors occurring continuously and apart from cell division.[5]

In conclusion, there is evidence in the literature to support the hypothesis that the formation of DNA bases, like that of the phosphorus moiety, takes place exclusively during the duplication stage of mitosis. However, the behavior of various components of DNA in relation to mitosis can not be definitely elucidated until the rates of formation of various DNA moieties have been estimated with precision.

Renewal of DNA in Relation to Mitosis

It has been shown above that formation of DNA phosphorus (DNA P) occurs exclusively during the duplication stage of the mitotic cycle. Since at this stage the basal amount of DNA is doubled prior to cell division, the amount of DNA P must also be doubled. Thus, before a mitosis occurs, the tissue content of DNA P is increased by an amount equal to the basal content of a cell; and, at mitosis, the number of cells in the tissue is also increased by one unit. Therefore, additional DNA P appears in a tissue as fast as new cells are formed. It had been naturally assumed that this additional DNA P accounted for all the new DNA P appearing during the duplication stage. To verify this conclusion, the rate of formation of DNA P was measured with the aid of P^{32}, and compared with the rate of cell formation. This comparison was made with liver, small intestine (50), and lung (13) of the rat. In the *liver*, the rate of cell formation was calculated from the number of nuclei per gram and the rate of increase in the weight of the organ. The daily increment in the number of cells was 0.71% of the whole cell population of the liver. For the mucosa of the small intestine—where there is an active and constant cell renewal (24)—the use of the colchicine technique permitted estimation of the rate of cell formation. Rats were given an injection of colchicine and sacrificed four hours later. Thus, it was found that 7·13% of the cells were arrested at metaphase, i.e. presumably all the cells which had entered mitosis in that four-hour period had been arrested by colchicine. Theoretically, six such experimental periods would result in 42·8% of the cells being in similar metaphase arrest. This figure, then, would represent the proportion of cells formed per day. (Correction for the time between the injection of colchicine and the effect of the drug in the tissue brings the

[4] *This conclusion is derived from the fact that agreement has been obtained with labeled adenine, ammonia, and phosphorus with respect to the DNA:RNA isotope content ratios using normal liver (1, 18, 19, 42), regenerating liver (18), and spleen and intestine (42). This may be taken to support the idea that adenine and ammonia, like phosphorus, are incorporated into DNA during the duplication stage. Data obtained with labeled glycine also agree with the P^{32} observations in the case of the intestine (1, 45).*

(The data obtained on pooled organs are not considered since they cannot be interpreted in relation to mitotic activity.)

[5] *Following administration of labeled glycine and formate to rats and mice, the DNA:RNA ratio in the liver was found to be much higher than the ratios obtained with P^{32} or labeled adenine as precursors (1, 2, 3, 18, 21, 30, 31, 42). Since liver has a low mitotic activity, it was concluded that the parts of the DNA molecule (bases) derived from glycine and formate are renewed in non-dividing cells.*

proportion of cells formed per day up to 54·3%.) Using the colchicine technique with *lung* tissue, 3·57% of the cells were found to undergo mitosis each day (8). (Corrected for the time lag of colchicine, the figure is 4·08 (Table V).)

The percentage DNA P formed in each tissue during various time intervals was estimated by comparing the specific activity of DNA at the moment the animal was sacrificed with the mean specific activity of the phosphorus precursor between the time of injection of P^{32} and the time of sacrifice. It was assumed that the immediate DNA precursors, presumably nucleotides, are components of the acid-soluble fraction (2, 42). Since free mononucleotides, isolated by ion exchange chromatography from the acid-soluble fraction of rat liver, had approximately the same specific activity as the whole fraction, it was concluded that mononucleotides were in rapid equilibrium with other acid-soluble phosphorus compounds (Daoust and Cantero, unpublished). Therefore, the values for the whole acid-soluble phosphorus were considered to be representative of those of the precursor for the purpose of the subsequent calculations. The daily formation of DNA P as calculated from the results obtained for various time intervals (Table V) was found to be 1.24% for the liver, 114.5% for the intestinal mucosa, and 10.66% for the lung.

If tissue inorganic phosphorus alone was considered to be the DNA precursor, the calculated values for the daily formation of DNA P become 1.13, 95.0, and 8.00% respectively. (Since the phosphorus present in the intermediates of DNA synthesis is necessarily derived from the inorganic phosphorus, and is thus of lower specific activity than the latter at early intervals after injection, the figures obtained for the daily formation of DNA P by using inorganic phosphorus as precursor may be considered to be minimum values.)

A comparison of the formation rates of cell and DNA P shows that in the liver, 0·71% of the cells were added each day, while 1·24–1·13% of the DNA P present was synthesized during the same time interval. In the intestinal mucosa, the daily formation of cells was found to be 54·3% (corrected value),

TABLE V

COMPARISON OF THE RATE OF FORMATION OF DESOXYRIBONUCLEIC ACID PHOSPHORUS WITH THE RATE OF CELL FORMATION IN THE LIVER, LUNG, AND SMALL INTESTINE OF THE RAT

	Per cent daily formation of DNA P		Per cent daily formation of cells
	Acid-soluble P used as precursor	Inorganic P used as precursor	
Liver (50)	1·24	1·13	0·71
Lung (13)	10·66	8·00	3·57 (4·08 corrected)
Intestinal mucosa (50)	114·5	95·0	42·8 (54·3 corrected)

while the daily formation of DNA P was 114·5–95·0%. In the lung, 4·08% (corrected value) and 10·66–8·00% were obtained for the daily formation of cells and DNA P respectively. In these three tissues, therefore, approximately twice as much new DNA P appeared as had been expected from the number of new cells (Table V). Hevesy and his collaborators observed similarly that in growing tissues, the amount of labeled DNA P formed during a given time interval was about twice that of the DNA P added to the organ during the same interval (22).

These results imply that during the stage of DNA synthesis, the amount of newly-formed DNA P must be twice that of the DNA P added. The basal amount of DNA P present during the stable stage of the interphasic period must, in the course of the duplication stage, be replaced by twice its amount of new DNA P. In other words, all the phosphorus must be new in the DNA of the cells arising from a mitosis.

Owing to its backbone position, replacement of the phosphorus in DNA can hardly be limited to the renewal of the phosphate group. Rather, such a renewal would suggest an extensive transformation of the "old" DNA molecules, possibly implicating the bases and carbohydrate moieties as well. If, as indicated above, it is assumed that other DNA components behave like the phosphorus moiety, the duplication stage may be redefined as being the time during which the amount of DNA present at the stable stage is demolished and replaced by twice as much new DNA.

It would appear, therefore, that mitosis is associated not only with the formation of additional DNA, but also with a complete replacement of all the DNA of the parent nucleus, so that only newly-formed DNA is present in the two daughter nuclei.

French and co-workers (17) independently observed that phage DNA phosphorus is not transmitted quantitatively to progeny. The results obtained by these authors might be explained by our observations, since in the course of formation of phage or virus DNA, there may be a replacement of each parent molecule by two new or daughter molecules.

Although it is not easy to visualize how the rigid chromosome pattern is maintained under these conditions, it is imperative that the biochemical dynamism of the genic material (during a given period of the mitotic cycle) be compatible with the transfer of hereditary characters.

Interpretation

The only well confirmed function of the cell nucleus is the maintenance of the hereditary pattern of the cell, but there is as yet no real information as to how this function is performed (34). The data presented here relate only to the morphology and chemical constitution of the nuclear material, its property of reproducibility at cell division, and the synthesis of the different moieties of DNA in respect to the various stages of the mitotic cycle.

Since the chromosomes appear to be identical in form at each cell division, it has been postulated that they exist throughout the life of the cell and there

is strong evidence to support this theory. We have seen that DNA is intimately concerned with the structure of the mitotic chromosome, and is actually located in the same positions as the carriers of hereditary factors, the genes. This is probably true of the interphase chromosomes as well, since DNA is present in constant amounts throughout the stable stage of the interphase life of the cell. The duplication of DNA during mitosis was shown to take place before the onset of visible prophase. Consequently, the interphase has been subdivided into three stages, namely, the "stable stage", "duplication stage", and "postduplication stage", depending upon the amount of DNA present in the nucleus.

From studies with radioisotopes, it was concluded that formation of DNA P occurs exclusively during the duplication stage of the mitotic cycle. During all other stages, the phosphate, and by inference, the sugar–phosphate backbone of DNA, remain stable. Moreover, there is some evidence to support the view that, in this respect, the purines and other components of DNA also remain stable.

However, the data presented show that twice as much DNA P is formed at any duplication stage than is required for the addition of one cell. This is taken to indicate that the phosphorus present in the DNA at the stable stage is lost at the duplication stage to be replaced by twice its amount of new phosphorus. It appears likely that not only the phosphorus component but the whole DNA molecule is lost. Thus, each DNA molecule would be replaced by two new ones, and the two daughter cells arising from mitosis would receive newly-formed DNA exclusively.

DNA is generally considered to be the chief chemical constituent of the genes. The DNA molecule, composed as it is of many nucleotides which may assume an infinite variety of spatial arrangements, seems well suited to provide the specific molecular pattern for each gene. However, the protein component of the nucleoprotein may also be necessary for the function and reproduction of the genes. When the chromosomic material is duplicated, the protein component may form the basic pattern or "template" which determines the design of the new chromosomal material. The other possibility is that the new DNA is patterned after itself, with old DNA molecules disappearing as new ones are formed. At any rate, it seems likely that the duplication of chromosomal DNA proceeds by a replacement of each of the old or parent molecules by two new, or daughter, molecules, and it has been suggested that this finding may also apply to the duplication of phage and virus DNA. That such a process is involved in the autoduplication of nucleoproteins in general appears a tempting hypothesis.

These various investigations, while bringing valuable information concerning gene reproduction, still throw no light on the mechanism by which the genes—and the associated DNA—control the hereditary characters. This appears to be the task for the future.

References

1. ABRAMS, R. Arch. Biochem. & Biophys. 33 : 436. 1951.
2. ABRAMS, R. and GOLDINGER, J. M. Arch. Biochem. & Biophys. 30 : 261. 1951.
3. ABRAMS, R. and GOLDINGER, J. M. Arch. Biochem. & Biophys. 35 : 243. 1952.
4. AHLSTRÖM, L., EULER, H. VON, and HEVESY, G. Arkiv Kemi Mineral. Geol. A, 19 : No. 9. 1944.
5. ALFERT, M. J. Cellular Comp. Physiol. 36 : 381. 1950.
6. ASTBURY, W. T. Symposium Soc. Exptl. Biol. 1 : 66. 1947.
7. BENDICH, A. Exptl. Cell Research, Suppl. 2 : 181. 1952.
8. BERTALANFFY, F. and LEBLOND, C. P. Anat. Record, 115 : 515, 1953.
9. BOIVIN, A., VENDRELY, R., and VENDRELY, C. Compt. rend. 226 : 1061. 1948.
10. BROWN, G. L., CALLAN, H. G., and LEAF, G. Science, 165 : 600. 1950.
11. CLAUDE, A. and POTTER, J. J. Exptl. Med. 77 : 345. 1943.
12. d'ANGELO, E. G. Ann. N.Y. Acad. Sci. 50 : 910. 1950.
13. DAOUST, R., BERTALANFFY, F., and LEBLOND, C. P. Unpublished results.
14. DAVIDSON, J. N. and LESLIE, I. Cancer Research, 10 : 587. 1950.
15. DODSON, E. O. Univ. Calif. Pub., Zoöl. 53 : 281. 1949.
16. DURYEE, W. R. Ann. N.Y. Acad. Sci. 50 : 920. 1950.
17. FRENCH, R. C., GRAHAM, A. F., LESLEY, S. M., and VAN ROOYEN, C. E. J. Bact. 64 : 597. 1952.
18. FURST, S. S. and BROWN, G. B. J. Biol. Chem. 191 : 239. 1951.
19. FURST, S. S., ROLL, P. M., and BROWN, G. B. J. Biol. Chem. 183 : 251. 1950.
20. HARRISON, M. F. Nature, 168 : 248. 1951.
21. HEIDELBERGER, C. and LEPAGE, G. A. Proc. Soc. Exptl. Biol. Med. 76 : 464. 1951.
22. HEVESY, G. Radioactive indicators. Interscience Publishers, Inc., New York, 1948.
23. HOWARD, A. and PELC, S. P. Exptl. Cell Research, 2 : 178. 1951.
24. HOWARD, A. and PELC, S. P. Nature, 167 : 599. 1951.
25. HUGHES, A. The mitotic cycle. Academic Press Inc., New York. 1952.
26. HULL, W. and KIRK, P. L. J. Gen. Physiol. 33 : 335. 1950.

26*a*. HUSKINS, C. L. and SMITH, S. G. Ann. Botany, 49 : 119. 1935.

27. JOHNSON, R. M. and ALBERT, S. Arch. Biochem. & Biophys. 35 : 340. 1952.
28. LEBLOND, C. P. and ALLEN, E. Endocrinology, 21 : 455. 1937.
29. LEBLOND, C. P. and STEVENS, C. E. Anat. Record, 100 : 357. 1948.
30. LEBLOND, C. P., STEVENS, C. E., and BOGOROCH, R. Science, 108 : 531. 1948.
31. LEPAGE, G. A. and HEIDELBERGER, C. J. Biol. Chem. 188 : 593. 1951.
32. LISON, L. and PASTEELS, J. Compt. rend. soc. biol. 143 : 1607. 1949.
33. LISON, L. and PASTEELS, J. Arch. biol. 62 : 2. 1951.
34. MAZIA, D. *In* Modern trends in physiology and biochemistry. *Edited by* E. S. Guzman Barron, Academic Press Inc., New York. 1952. p. 77.
35. MIRSKY, A. E. and RIS, H. J. Gen. Physiol. 31 : 1. 1947.
36. MIRSKY, A. E. and RIS, H. J. Gen. Physiol. 31 : 7. 1947.
37. MIRSKY, A. E. and RIS, H. Nature, 163 : 666. 1949.
38. OSGOOD, E. E., LI, J. G., TIVEY, H., OVERST, M. L., and SEAMAN, A. L. Science, 114 : 95. 1951.
39. PASTEELS, J. and LISON, L. Compt. rend. 230 : 780. 1950.
40. PASTEELS, J. and LISON, L. Arch. biol. 61 : 445. 1950.
41. PASTEELS, J. and LISON, L. Compt. rend. 233 : 196. 1951.
42. PAYNE, A. H., KELLY, L. S., and JONES, H. B. Cancer Research, 12 : 666. 1952.
43. PELC, S. P. and HOWARD, A. Brit. Med. Bull. 8 : 132. 1952.
44. PRICE, J. M. and LAIRD, A. K. Cancer Research, 10 : 650. 1950.
45. REICHARD, P. J. Biol. Chem. 179 : 773. 1949.
46. RIS, H. and CROUSE, H. Proc. Natl. Acad. Sci. U.S. 31 : 321. 1945.
47. SESHACHAR, B. R. Nature, 165 : 848. 1950.
48. SPARROW, A. H. Can. J. Research, C, 20 : 257. 1942.
49. STERN, K. G. Yale J. Biol. and Med. 19 : 937. 1947.
50. STEVENS, C. E., DAOUST, R., and LEBLOND, C. P. J. Biol. Chem. 202 : 177. 1953.
51. STOWELL, R. E. Stain Technol. 21 : 137. 1946.
52. SWIFT, H. H. Physiol. Zoöl. 23 : 169. 1950.
53. VANDERLYN, L. Science, 104 : 514. 1946.
54. VANDERLYN, L. Botan. Rev. 14 : 270. 1948.
55. VENDRELY, R. and VENDRELY, C. Experientia, 4 : 434. 1948.
56. VENDRELY, R. and VENDRELY, C. Experientia, 5 : 327. 1949.
57. WATSON, M. L. University of Rochester Atomic Energy Project, Unclassified report—U.R.-185, Rochester, N.Y. 1952.

DISCUSSION

Q.—**Dr. W. R. Phillips** (*Central Experimental Farm, Ottawa*): Do nuclei of classes II and III undergo the same type of mitosis as those of type I?

A.—**Dr. Stevens:** The general process of mitosis appears to be the same for nuclei of classes II and III as for those of class I, but the number of chromosomes present is two or four times respectively as high as in class I.

Q.—**Dr. D. B. Smith** (*National Research Council, Ottawa*): Does the DNA content of the cell remain constant throughout the visible mitotic changes through to telophase when the new nuclei are formed, and does this amount double during the resting stage?

A.—**Dr. Stevens:** Yes, in most cases. There is an exception in the case of rapidly dividing cells, such as those of the crypts of Lieberkühn of the rat, in which the synthesis of DNA for the *next* mitosis occurs at telophase (see text).

Q.—**Dr. W. Andreae** (*Science Service Laboratory, London, Ont.*): Is there any information as to how one gene differs chemically from another?

A.—**Dr. Stevens:** There is little solid information on the subject. Variations in the respective numbers of the various bases is the most widely quoted possibility. A discussion of the subject may be found in Stern's review (49).

Comment—**Dr. Zamenhof:** Thus far nothing is known about these chemical differences.

THE VIRUSES AS NUCLEOPROTEINS[1]

By A. F. Graham

Abstract

A review of work in the bacteriophage field is presented. The discussion is concerned mainly with biochemical aspects of the problem but some reference is also made to genetic studies.

Introduction

When a virus is brought into contact with a susceptible cell a process is initiated which normally leads to the production of more virus. Not only is the presence of the living cell an essential for viral growth, but the viruses themselves are highly specific in their choice of host. A wide range of viruses is known to attack insects, plants, animals, bacteria, and actinomycetes, and often the invasion is confined to one particular type of cell. To take an example, it is not uncommon to find a bacterial virus which will attack one strain of *E. coli* but not another. An interesting feature of the polyhedral insect viruses is that they attack only the larval stages whereas the adult insects are apparently immune (72).

The viruses are discrete particles with definite morphology as can be seen in the electron microscope. In size, they range from 10 to 300 mμ in maximum diameter and often vary considerably in particle shape. The Newcastle virus of chickens is spermlike in shape as are many bacterial viruses. The rabbit papilloma virus and some plant viruses are small spheres while other plant viruses are long rods.

The chemical composition is known for relatively few viruses (6, 7). Before accurate chemical analysis can be performed there must be assurance that the preparation consists only of virus. In the case of animal viruses the separation of virus from cell components is particularly difficult since particles are normally present in the cell of about the same size as the virus. Moreover, the concentration of virus in animal tissue is usually relatively low. On the other hand it seems fairly easy to obtain large amounts of some plant viruses in crystalline form. Without exception those viruses which have been analyzed contain protein and nucleic acid. While we usually assume, therefore, that nucleic acid is a constituent of all viruses we should perhaps remember that this is a generalization from a small number of proved cases. The plant viruses contain ribonucleic acid (RNA) which may constitute up to 35% of their weight (67), the remainder being protein. The different bacterial viruses are very similar in composition, containing protein and up to 50% desoxyribonucleic acid (DNA). Animal viruses frequently have a more complex composition. In addition to protein they may contain RNA or DNA, or both together as in influenza, and they may contain up to 50% lipid material, as in equine encephalomyelitis. The composition of the virus

[1] *Manuscript received January 27, 1953.*
Contribution from Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario.

nucleic acid may differ markedly from that of the host. For example, it has been shown recently that the relative proportions of purine and pyrimidine bases in the DNA of several insect (76) and bacterial viruses (71, 77) are unlike those of the respective hosts. Indeed the coliphages T2, T4, and T6 have been found to contain considerable amounts of a new pyrimidine, 5-hydroxymethyl cytosine, which is absent entirely from *E. coli* (77).

The chemical and morphological complexity, particularly of some of the larger animal viruses, suggests structures with a fairly high degree of organization. Nevertheless, a great deal of work on purified viruses has failed to demonstrate the presence of enzymes, with the possible exception of a mucinase in influenza (4, 5). Apparently, then, the viruses have little or no independent metabolism and we are faced with the very intriguing question as to how they multiply in the host cell.

For many years it was considered that viruses invade the cell and multiply there by binary fission like bacteria. It should be realized, however, that cellular multiplication itself involves much more than mere division. The vital phase of multiplication, that is the duplication of existing enzymes, nucleic acids, and so forth, has already been completed by the time the cell divides. In effect fission represents the separation of two already organized structures. The real issue is to determine how this state of organization is achieved; how the various metabolites are brought together to endow the constituents of the cell with their unique biological specificities. In so far as the viruses are nucleoproteins the study of virus growth resolves itself to a study of the synthesis of nucleic acid and protein.

In order to discuss this problem in more detail let us consider a particular set of viruses, the bacterial viruses or bacteriophages—usually abbreviated to phages. A large amount of information has been gathered about their biological behavior since these viruses are easier to handle than any other virus group (1). There is reason to believe also that the general picture, being developed from bacteriophage work, probably applies to other virus systems and in this respect the phage system serves as a useful experimental model.

The phages with which we are best acquainted are those we shall call the virulent phages. These invade the cell, multiply within, and finally cause destruction of the cell. This sequence has its counterpart in many of the animal and insect virus infections. Not all phages cause this clear-cut picture of infection. The recent studies of Lwoff and his co-workers in Paris (43, 62, 63, 64, 65) have revived interest in the temperate phages which cause the cell to become lysogenic. If a culture of sensitive cells, for example *B. megatherium*, is infected with a temperate phage most of the cells lyse in the usual manner. A small percentage of the cells receive the virus but are not destroyed. In these cases the infection has been an abortive one and the cells to all appearances are normal. They contain no virus and can grow and divide indefinitely as usual. Nevertheless they are unstable in that they have a tendency, inherited from one generation to another, to complete

the cycle and form phage, and so are called lysogenic. This instability is manifested in one obvious way; namely in a growing lysogenic culture a very small proportion of the cells, less than 1 per 1000, do lyse spontaneously and liberate virus. These cells have thus completed the cycle of infection. Some lysogenic cultures possess a very striking property; if the culture is given a small dose of ultraviolet radiation all the cells in the culture will burst within 60 min. and produce about 100 new virus particles each. This new virus is identical with that which produced the lysogenic cells originally, perhaps many hundreds of cell divisions before. Exposure to ultraviolet light is termed induction and the ensuing period of virus growth is referred to as the latent period. The lysogenic cells can also be induced to form virus by the action of many reducing agents such as thiomalate and ascorbic acid. Whether the cells can be induced or not by any of these agents depends to a very marked extent on the composition of the nutrient medium. Also it should be mentioned that not all lysogenic strains can be induced, or at least the most effective stimuli for induction have yet to be found.

Lwoff has suggested that the lysogenic bacteria perpetuate a specific particle, the prophage, which can be reproduced through successive generations of the cell, is nonpathogenic, but is potentially lethal. Thus the genetic factor which predisposes the cell to produce phage may be carried in the prophage and the unstable equilibrium can be upset at will in the direction of complete phage formation by the inducing agent. It is an attractive possibility that the prophage may, in fact, be part of the chromosomal structure of the lysogenic bacterium.

The biological significance of this phenomenon, both from the fundamental and practical point of view, is enormous. The nature of the prophage and the mechanism of induction are as yet unknown. However, behavior analogous to lysogenicity, or latent infection, is recognized with animal viruses, for example, recurrent herpes labialis (73), and in particular in the insect viruses (9). Probably the phenomenon is far more widespread in virus infection than we yet realize and in fact a strong case may be made for the concept that viruses survive by such a mechanism (10). The induction effect may also be likened to the action of ultraviolet light and chemical carcinogens in their capacity to upset the stability of mammalian and avian cells in the direction of malignancy. The discovery of the effect in lysogenic bacteria offers an ideal opportunity to study its mechanism under controlled experimental conditions.

Let us now consider some of the biochemical changes which take place in the virus infected cell. The discussion will be confined mainly to the virulent phages T2, T4, and T6, active on the bacterial host *Escherichia coli*, which are similar in properties and behavior. They may be obtained readily in quantity in a high state of purity by a differential centrifugation procedure (35). They are tadpole-like in shape with heads roughly 80 mμ in diameter and tails 100 $\times$ 20 mμ. Their composition is about 60% protein and

40% DNA and from present evidence it seems likely that the DNA is packed into the center of the head and there held by the surrounding protein membrane (34).

The first step in infection is the adsorption of the virus to its host. The particle seems to adhere to the cell by its tail (2) and the efficiency of this adsorption is greatly dependent upon the type and concentration of ions in the medium (31, 69). Only part of the virus penetrates into the cell. This has been shown in a very striking manner by Hershey and Chase (40) for T2 coliphage. The phage was labelled in the protein moiety with S^{35}. When the cell was infected with this phage it was then subjected to strong shearing force in a Waring blendor and 80% of the S^{35} was removed from the cell surface. Notwithstanding this drastic treatment the cell went on to produce its yield of phage in the normal way. Thus, once infection was initiated, most of the sulphur-containing protein of the invading phage took no further part in the intracellular production of new virus. Studies with P^{32} labelled phage have shown that most if not all of the phage DNA penetrates into the cell and there is reason to believe that it may be funneled into the cell through the tail of the adsorbed particle. Right at the outset of infection there is consequently an extensive separation of the components of the virus. There is probably also a further degradation of the phage nucleic acid immediately after entry into the cell.

During the next 23 min. the T2 phage multiplies inside the cell and this interval is designated the latent period, analogous to the interval following induction of lysogenic bacteria. Within a few minutes of infection an extensive intracellular rearrangement of chromatinic material occurs (60, 68). No infective particles can be found at this stage if the cells are ruptured artificially. These begin to appear at about 11 min. and increase rapidly in number until the cell lyses at the end of the latent period and releases 150 or more new phages (3, 21, 24).

Radiation techniques with ultraviolet and X rays have been utilized to gain information about events during the latent period (51, 52, 61). Thus if free phage is irradiated the curve for survival of infectivity against dosage follows an exponential or "one-hit" law. If cells are infected with single phage particles and irradiated immediately after infection the same one-hit type of survival curve should be obtained. As the virus develops intracellularly a multiple hit curve should result, the multiplicity being equivalent to the number of intracellular phages. When this method is applied to the small spherical coliphage T7 the results are similar to those predicted by target theory (8). The resistance of the intracellular phage to radiation is the same as that of free phage as shown by the slopes of the survival curves. However, it is found that the transition from single to multiple hit curves occurs before infective particles can be found inside the cell. When the same techniques are applied to T2 infection anomalous results are obtained and the picture is a great deal more complex. It has therefore been suggested that there may be large differences in the modes of reproduction of different phages (8).

Recent electron microscope work (53, 58) has revealed that a number of round, tailless, noninfectious particles appear in the cell during infection with T2 phage. These have been obtained from prematurely lysed cells and from cells in which growth of mature phage was inhibited by proflavine (29). When purified by centrifugation they were found to consist mainly of protein; little DNA was present but it is not yet known whether the particles are associated with DNA inside the cells (53). It has been suggested (58) that they may be the immediate precursors of the virus which are rapidly replaced by the final tailed infectious units. From the radiation and microscope studies it would seem, therefore, that multiple entities appear fairly early in the infected cell and that the property of infectiousness might be acquired by these at a late, if not the last, stage in the production of virus.

Infection with the virulent phages immediately prevents further cell division. There is no further increase in respiratory enzymes and adaptive enzyme formation is arrested. Normally there is three to five times as much RNA as DNA in the host. Infection of the host stops RNA synthesis and isotope studies have shown that there is not even turnover of this substance. Nor is there turnover of phospholipid phosphorus. Protein is synthesized from the start of infection. This behavior (11, 14, 15, 16) is in contrast to that of lysogenic bacteria (62, 63). After induction with ultraviolet radiation the cells continue to grow and divide through most of the latent period. RNA synthesis and formation of respiratory enzymes parallel cell growth. Also adaptive enzymes can be formed during the latent period. Comparing the two systems, the synthesis of RNA and formation of respiratory and adaptive enzymes seem to depend on whether or not the cell can grow during the period of intracellular virus multiplication. In this respect the virulent phages have complete domination over the metabolism of the infected cell. However, it should be emphasized that with both induced lysogenic strains and cells infected with virulent phage there is first a marked lag in DNA formation followed by synthesis at a greatly accelerated rate (12, 63). Thus the most apparent aberration of nucleic acid metabolism during phage growth is the initiation of new and abnormal DNA synthesis.

In view of these observations it is apparent that the invading virus does not enter the cell and grow there as a particulate entity. Aside from the fact that it exhibits no independent metabolic activity it is known that the particle is extensively broken down after invasion. We must conclude that the ensuing synthesis of new virus is carried out by the metabolic apparatus of the cell itself. It seems that the reactions of the cell are diverted into new pathways by the presence of the virus. A transformation has occurred resulting in reorganization of the cell's structure and a shift in its metabolic equilibrium. Thus we conceive the process of infection as one in which both the virus and the cell lose their original identity. They are replaced by a new entity, the virus infected cell, in which the pre-existing metabolic reactions are now under a genetic control imposed by the virus. The predominant function of this complex is the synthesis of nucleoprotein for the construction of new virus.

Let us next discuss the origin of the building blocks utilized in the formation of virus nucleoprotein and here we shall take as an illustrative model the virulent coliphages T2, T4, and T6. It should be noted that most of the DNA, and probably protein as well, formed after infection appears in the new virus. Isotope studies have shown that about 30% of the phosphorus (13, 49, 50) and 20% of the nitrogen (28, 48), required for this synthesis, are derived from constituents present in the cell before infection. The remainder of these elements is taken from inorganic constituents in the nutrient medium. The greater part of these viruses is therefore synthesized *de novo*.

As yet there is little information about the synthesis of the protein moiety but the formation of virus nucleic acid is being intensively studied. Both the purines and pyrimidines of the host cell can be labelled selectively with C^{14} (27, 44, 45, 74). If the cell is then infected with T2 or T6 phages these bases may be transferred as such to the virus DNA. Since it has been found that there is no turnover of phosphorus or purines in cellular RNA after infection (13, 45) the bases contributed to the phage must originate from host DNA. On this assumption it has been found that about 100% of the adenine and 75% of the guanine of cellular DNA are incorporated into phage DNA (45). No other nitrogenous compound of the host is utilized for the synthesis of phage purines. It is also known that if infected cells are placed in a medium containing labelled adenine or guanine these compounds will be built into the phage structure although some interconversion of the two bases may occur (45).

It might be thought that the nucleic acid of the host cell which appears in the phage carries some specific biological function from the cell but such does not appear to be the case. There is good evidence that the cellular DNA is broken down at least to the nucleotide stage after infection before being built up again into virus material (28, 74). Further the cell contains only about one-third the quantity of DNA required for T2 phage synthesis. This supply is exhausted early and its pyrimidines and purines appear only in the early particles formed in the cell. The DNA of later particles seems to be synthesized from carbon sources in the medium. If there is anything specific about the transfer of host material one would think that it should be distributed evenly over all the viral progeny.

Up to this point I have been drawing largely on biochemical evidence to describe the changes in the infected cell but I should like here to refer to a few salient observations in the field of phage genetics. In considering the mechanism of phage growth we should remember that practically all the new particles formed in the cell are identical with the infecting particle in genetic as well as biochemical properties. In a small number of cases, however, mutant forms appear in the progeny. These mutants are closely related to the infecting particle but can be differentiated by certain well recognized properties (36, 37). On infection with the mutant forms the mutant character will carry through into the progeny. The infecting virus has, in fact, been labelled at a specific point with a genetic marker.

Luria (57, 58) has made an analysis of spontaneous phage mutations occurring in individual cells infected with T2 phage. The mutants occur in clones and the distribution of the number of mutants per clone supports the assumption that intracellular phage growth occurs by a method formally analogous to binary fission. Yet the genetic (22, 58) and biochemical (21, 24, 40) evidence points to an extensive breakdown of the infecting phage. Therefore it seems necessary to postulate that at some stage in their development the newly growing phages must in turn act as models or replicas for the formation of more particles.

The study of phenomena attending infection of the cell with two different viruses has provided a fruitful field for phage geneticists. Thus it is well known that if the *E. coli* cell is infected simultaneously with two unrelated T phages only one or the other will grow but not both (17, 18, 19, 26, 75). This is referred to as the "interference effect" or "mutual exclusion", and while I shall not discuss it further here (32), it should be pointed out that the effect is not confined to phages and is of great practical and theoretical importance in viral infection (33). If the host is infected with two T phages which are closely related, such as T2 and T4, both may grow and produce progeny (20). Both these viruses can be labelled with specific genetic markers which we shall call r and r^+. When the cell is then infected simultaneously with a pair of viruses such as T2r^+ and T4r the resulting progeny yield will contain the two parental forms as well as two new types T2r and T4r^+ (38, 39, 42). There has thus been an exchange of genes between the two phages growing in the same cell. This very important effect is termed genetic recombination. When the mixed infection is carried out and the cells are prematurely lysed at various times it is found that the earliest phages formed contain almost as many recombinant particles as the later phages (22, 23). These recombinants are distributed very nearly at random in individual bacteria. Genetic recombination must therefore be a later stage in viral reproduction than mutation, otherwise the recombinants would appear in clones (55, 56). Further it may be concluded that the first complete phage particles which appear in the cell are not the parents of later particles (22).

A somewhat different type of interaction is known as multiplicity reactivation (54, 59). Thus if a phage is irradiated with ultraviolet it may absorb to the host and kill the cell but no intracellular growth of new virus will result. If two or more of the inactivated particles adsorb to the same cell the cell may become infected and produce a normal yield of phage. The early interpretation placed on this result (59) suggested that the phage contained a number of discrete genetic subunits which could reproduce individually within the cell. Irradiation damaged some of these units and inactive virus could not infect the cell until genetic recombination between a number of inactivated particles brought together a complete set of undamaged units. The early results have recently been extended (25, 58) and it appears that the explanation based on genetic recombination can no longer account for the facts. At present we are without a satisfactory explanation for this phenomenon.

Returning to the more biochemical aspects we have yet to consider what material contribution the infecting particle makes to its progeny. It seems that the genetic specificity of the phage may be largely a property of its nucleic acid and it is a problem of the greatest importance to determine how this specificity is transferred to the new phage. It is known from isotope studies that about 15% of the nitrogen (46) and 35% of the DNA phosphorus (70) in the invading particle appear in the progeny. It would seem a fair assumption that this transferred material contains the stable genetic core but present evidence is opposed to this view. It has been shown that the first generation progeny containing 35% of parental phosphorus itself transmits 35% of its phosphorus to progeny when used to infect fresh cells (41, 66). This indicates that there is no stable chemically intact core in the phage which passes through successive generations. Also the parental phosphorus is apparently contained only in the first progeny particles formed (30). Since the genetic properties of early and late progeny particles are the same it would thus appear that the transferred phosphorus is not associated with genetic material. Further study of phage labelled with N^{15} and P^{32} indicates that the parental material is passed on to the progeny in random fashion (47). Increasing evidence suggests, therefore, that the parental material may be degraded to a nonspecific level before incorporation into the growing phage.

From the weight of evidence adduced it seems that we are led to the following general conclusions. The genetic specificity does not depend upon any material which the growing virus utilizes from either host or infecting particle. It seems rather that building blocks for the synthesis may be taken indiscriminately from any available source. The specificity arises entirely from the direction imposed upon the cellular metabolism at the moment of infection.

While excellent progress has been made during the past few years many important questions remain to be answered. We have no idea how the infecting particle assumes control in the cell; the mechanism by which the cellular enzymes are organized is unknown; nor do we know how prophage is induced to become infectious phage; how or at what point the phage components are assembled into infectious units; or how the genetic mixing for recombination occurs. These problems could be multiplied indefinitely. But my main purpose in this paper has been to present to you a general picture of the present state of the virus field from a biochemist's point of view and to point out that the study of viruses is not limited to the aspects of disease alone but embraces many fundamental problems in biology.

Acknowledgment

The original work of the author and his co-workers Dr. R. C. French, Dr. C. E. van Rooyen, and Mr. S. M. Lesley, has been supported by grants from the National Cancer Institute of Canada; the National Institutes of Health of the United States, Public Health Service; and the W. B. Boyd Memorial Fund of the Connaught Medical Research Laboratories.

References*

1. ADAMS, M. Methods in medical research. Vol. 2. The Year Book Publishers, Inc., Chicago. 1950.
2. ANDERSON, T. F. Am. Naturalist, 86 : 91. 1952.
3. ANDERSON, T. F. and DOERMANN, A. H. J. Gen. Physiol. 35 : 657. 1952.
4. BAUER, D. J. Nature, 164 : 767. 1949.
5. BAUER, D. J. Symposium on the nature of virus multiplication. Soc. Gen. Microbiol. In press. 1952.
6. BEARD, J. W. J. Immunol. 58 : 49. 1948.
7. BEARD, J. W. Ann. Rev. Microbiol. 5 : 265. 1951.
8. BENZER, S. J. Bact. 63 : 59. 1952.
9. BERGOLD, G. H. Private communication.
10. BOYD, J. S. K. Symposium on the nature of virus multiplication. Soc. Gen. Microbiol. In press. 1952.
11. COHEN, S. S. Cold Spring Harbor Symposia Quant. Biol. 12 : 35. 1947.
12. COHEN, S. S. J. Biol. Chem. 174 : 281. 1948.
13. COHEN, S. S. J. Biol. Chem. 174 : 295. 1948.
14. COHEN, S. S. Bact. Revs. 13 : 1. 1949.
15. COHEN, S. S. Bact. Revs. 15 : 131. 1951.
16. COHEN, S. S. Ann. N. Y. Acad. Sci. 54 : 902. 1952.
17. DELBRÜCK, M. J. Bact. 50 : 151. 1945.
18. DELBRÜCK, M. Biol. Revs. 21 : 30. 1946.
19. DELBRÜCK, M. Harvey Lectures, 41 : 161. 1946.
20. DELBRÜCK, M. AND BAILEY, W. T., Jr. Cold Spring Harbor Symposia Quant. Biol. 11 : 33. 1946.
21. DOERMANN, A. H. Carnegie Inst. Wash. Yearbook, 47 : 176. 1948.
22. DOERMANN, A. H. Carnegie Inst. Wash. Yearbook, 48 : 170. 1949.
23. DOERMANN, A. H. Federation Proc. 10 : 591. 1951.
24. DOERMANN, A. H. J. Gen. Physiol. 35 : 645. 1952.
25. DULBECCO, R. J. Bact. 63 : 199. 1952.
26. DULBECCO, R. J. Bact. 63 : 209. 1952.
27. EVANS E. A., Jr. Bact Revs. 14 : 210. 1950.
28. EVANS, E. A., Jr. Ann. N. Y. Acad. Sci. 54 : 909. 1952.
29. FOSTER, R. A. C. J. Bact. 56 : 795. 1948.
30. FRENCH, R. C., GRAHAM, A. F., LESLEY, S. M., AND VAN ROOYEN, C. E. J. Bact. 64 : 597. 1952.
31. GAREN, A. and PUCK, T. T. J. Exptl. Med. 94 : 177. 1951.
32. GRAHAM, A. F. Ann. inst. Pasteur. 84 : 90. 1953.
33. HENLE, W. J. Immunol. 64 : 203. 1950.
34. HERRIOTT, R. M. J. Bact. 61 : 752. 1951.
35. HERRIOTT, R. M. and BARLOW, J. L. J. Gen. Physiol. 36 : 17. 1952.
36. HERSHEY, A. D. Cold Spring Harbor Symposia Quant. Biol. 11 : 67. 1946.
37. HERSHEY, A. D. Genetics, 31 : 620. 1946.
38. HERSHEY, A. D. Cold Spring Harbor Symposia Quant. Biol. 16 : 471. 1951.
39. HERSHEY, A. D. Ann. N. Y. Acad. Sci. 54 : 960. 1952.
40. HERSHEY, A. D. and CHASE, M. J. Gen. Physiol. 36 : 39. 1952.
41. HERSHEY, A. D., ROESEL, C., CHASE, M., and FORMAN, S. Carnegie Inst. Wash. Yearbook, 50 : 195. 1951.
42. HERSHEY, A. D. and ROTMAN, R. Genetics, 34 : 44. 1949.
43. JACOB, F., SIMINOVITCH, L., and WOLLMANN, E. Ann. inst. Pasteur, 83 : 295. 1952.
44. KOCH, A. L., PUTNAM, F. W., and EVANS, E. A., Jr. J. Biol. Chem. 197 : 105. 1952.
45. KOCH, A. L., PUTNAM, F. W., and EVANS, E. A., Jr. J. Biol. Chem. 197 : 113. 1952.
46. KOZLOFF, L. M. J. Biol. Chem. 194 : 83. 1952.
47. KOZLOFF, L. M. J. Biol. Chem. 194 : 95. 1952.
48. KOZLOFF, L. M., KNOWLTON, K, PUTNAM, F. W., and EVANS, E. A., Jr. J. Biol. Chem. 188 : 101. 1951.
49. KOZLOFF, L. M. and PUTNAM, F. W. J. Biol. Chem. 182 : 229. 1950.
50. LABAW, L. W. J. Bact. 62 : 169. 1951.
51. LATARJET, R. J. Gen. Physiol. 31 : 529. 1948.
52. LATARJET, R. Symposium on the nature of virus multiplication. Soc. Gen. Microbiol. In press. 1952.
53. LEVINTHAL, C. and FISHER, H. Biochim. Biophys. Acta, 9: 419. 1952; and private communication from Dr. Levinthal.
54. LURIA, S. E. Proc. Natl. Acad. Sci. U.S. 33 : 253. 1947.

**References 5, 10, 52, 63, and 67 to "Symposium on the nature of virus multiplication" are to uncorrected proofs of papers circulated in April 1952 by the Society for General Microbiology.*

55. LURIA, S. E. Science, 111 : 507. 1950.
56. LURIA, S. E. Federation Proc. 10 : 582. 1951.
57. LURIA, S. E. Cold Spring Harbor Symposia Quant. Biol. 16 : 463. 1951.
58. LURIA, S. E. Symposium on the nature of virus multiplication. Soc. Gen. Microbiol. In press. 1952.
59. LURIA, S. E. and DULBECCO, R. Genetics, 34 : 93. 1949.
60. LURIA, S. E. and HUMAN, M. L. J. Bact. 59 : 551. 1950.
61. LURIA, S. E. and LATARJET, R. J. Bact. 53 : 149. 1947.
62. LWOFF, A. Endeavour, 11 : 72, 132. 1952.
63. LWOFF, A. Symposium on the nature of virus multiplication. Soc. Gen. Microbiol. In press. 1952.
64. LWOFF, A. and GUTMAN, A. Ann. inst. Pasteur, 78 : 711. 1950.
65. LWOFF, A., SIMINOVITCH, L., and KJELDGAARD, N. Ann. inst. Pasteur, 79 : 815. 1950.
66. MAALØE, O. and WATSON, J. D. Proc. Natl. Acad. Sci. U.S. 37 : 507. 1951.
67. MARKHAM, R. Symposium on the nature of virus multiplication. Soc. Gen. Microbiol. In press. 1952.
68. MURRAY, R. G. E., GILLEN, D. H., and HEAGY, F. C. J. Bact. 59 : 603. 1950.
69. PUCK, T. T., GAREN, A., and CLINE, J. J. Exptl. Med. 93 : 65. 1951.
70. PUTNAM, F. W. and KOZLOFF, L. M. J. Biol. Chem. 182 : 243. 1950.
71. SMITH, J. D. and WYATT, G. R. Biochem. J. 49 : 144. 1951.
72. SMITH, K. M. Endeavour, 10 : 194. 1951.
73. VAN ROOYEN, C. E. and RHODES, A. J. Virus diseases of man. Thomas Nelson & Sons, New York. 1948.
74. WEED, L. L. and COHEN, S. S. J. Biol. Chem. 192 : 693. 1951.
75. WEIGLE, J. J. and DELBRÜCK, M. J. Bact. 62 : 301. 1951.
76. WYATT, G. R. J. Gen. Physiol. 36 : 201. 1952.
77. WYATT, G. R. and COHEN, S. S. Nature, 170 : 1072. 1952.

DISCUSSION

Q.—**Dr. Zamenhof:** Is there any evidence that the DNA of the original infecting particle breaks down before it has a chance to duplicate? Is it not possible that this breakdown may occur after at least one duplication so as to ensure genetical continuity?

A.—**Dr. Graham** (*Connaught Medical Research Laboratories, Toronto*): We know that the phosphorus and purine in the DNA of the infecting particle are transferred with an efficiency of 40 to 50% to the progeny. The remainder of the DNA is retained in the cell and released on lysis in a degraded form. When the degradation occurs is unknown. While it would seem that these products are being discarded as waste material at the end of the growth process it is quite possible that earlier on, this part of the DNA plays some prominent role in the intracellular synthesis. Regarding the material transferred from parental DNA to new virus, present evidence indicates this is not carried over in special parts of the infecting particle. It has been contended that the transferred DNA is broken down into fragments before being incorporated nonspecifically into the progeny. I feel that this is far from proved and further experiments may yet show that there is a transfer of special units from the parent to ensure genetical continuity.

Q.—**Dr. R. J. Rossiter** (*University of Western Ontario*): What happens to the phosphorus of the host cell as the virus grows?

A.—**Dr. Graham:** About 10% of the bacterial phosphorus is incorporated into the virus. Apparently there is no turnover of RNA or phospholipid phosphorus. The major P contribution from host to virus is from cellular DNA, at least half the DNAP being used for this purpose.

Q.—**Dr. R. Gaudry:** Is the lipid component of many of the viruses an essential part of their structure?

A.—**Dr. Graham:** In some instances it may be. For example equine encephalomyelitis virus contains over 50% lipid and it has been claimed that the chemical composition is quite uniform from one preparation to another. As far as I know no method has been devised for reducing the lipid content without destroying the infectivity of the virus. It should be kept in mind, however, that the virus was obtained from infected chicken embryos, a starting material relatively high in fat, and part of the viral lipid might be a contaminant carried through the purification process. Rabbit papilloma virus and the coliphages appear to contain little or no fat.

SOME BIOLOGICAL EFFECTS OF IONIZING RADIATIONS AND THEIR EFFECTS ON DESOXYRIBONUCLEIC ACID[1]

By David B. Smith

Abstract

Some effects of ionizing radiation on mitosis are described. The effects of X- and γ-rays on desoxyribonucleic acid and investigations into the mechanism of these effects are reviewed.

Shortly after the discovery of X-rays, it became apparent that they had an adverse effect on the skin. Similar effects were found with β- and γ-rays from radioactive materials. Investigation disclosed that different tissues were damaged in varying degrees and that the tissues most affected were those in which cell division was most frequent. The first observable microscopic effect was a change in the chromosomes and the sequence of events that occur during cell division. Then in 1929, Muller found that X-rays would speed up the mutation rate greatly without necessarily changing the visible appearance of the chromosomes. It is now apparent that changes, seen later in tissues and organs, stem from these cytological changes. Since desoxyribonucleic acids are important nuclear constituents (15), the action of ionizing radiations on these substances and on the nucleoproteins with which the nucleic acids may be associated in the cell has been investigated.

The more common types of ionizing radiations are α-, β-, and γ-rays, artificially accelerated nuclei and electrons, and X-rays. Some of their characteristics are given in Table I. These radiations ionize atoms and resulting chemical and biological effects arise directly or indirectly from this ionization. Differences between the effects of the various radiations are explicable on the basis of ion density and distribution (10, 21). X-rays and γ-rays cause the ejection of a planetary electron from one or a few atoms. These secondary electrons are usually sufficiently energetic to produce many more ionizations among the atoms through which they pass before slowing down.

The voluminous literature on the biological effects of ionizing radiation is not easy to interpret, but on the cytological level the picture is a little clearer (16, 20). Investigation into the effects of these radiations on nuclear division (mitosis) in particular has been most fruitful.

The first observable effect of radiation is an inhibition of mitosis (4, 21). Cells in the process of division usually complete the cycle but further mitosis in the tissue is inhibited. The degree and duration of this inhibition are dependent on the amount of radiation given. With moderate doses mitosis is only delayed and tissue growth continues through increase in cell volume. During recovery, the frequency of mitosis is increased so that eventually the total number of mitoses approximates the number that would have occurred

[1] *Manuscript received January 27, 1953.*
Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa. Issued as N.R.C. No. 3010. This paper was read at the Nucleoprotein Symposium sponsored by the Chemical Institute of Canada at Laval University, Quebec, Que., October 9th, 1952.

had there been no irradiation. When irradiation of the nucleus is avoided, much higher doses are required for biological effects (16). These results indicate that the nucleus and the mitotic process are the most sensitive to irradiation and that the effects of ionizing radiation on other cell processes are of minor importance.

The effect of radiations on the nucleus was early attributed (23) to interference with nucleic acid metabolism. More recently, it has been shown that incorporation of C^{14} and P^{32} into desoxyribonucleic acid is reduced by small X-ray doses (17, 18); in fact, disturbances in nucleic acid metabolism are the chief biochemical features of X-irradiation (16). Investigation of this interference with the production of an important constituent of the chromosomes may lead towards a better understanding of the mechanism of mitotic inhibition.

A clumping of chromosomes ("stickiness") in cells irradiated during certain stages of cell division is another effect of radiation (19). It has been suggested (14) that this "stickiness" is due to depolymerized desoxyribonucleic acid forming excess linkages between chromosomes. The exact nature of the phenomenon still awaits explanation.

After recovery from mitotic inhibition, various other abnormalities may be found at the next division. Some of these are incomplete spiralization of the chromosome, faulty spindle formation, deficiencies in the nucleic acid content of parts of chromosomes, and, most important and frequent, structural changes in the chromosomes. The latter have been extensively studied. These structural changes are due to breaks which must be caused, as argued

TABLE I

IONIZING RADIATIONS

Type	Primary particle	Ionizing mechanism	Source
α-Rays	Helium nucleus	Ejection of electron by secondary electrons	Radioactive materials
β-Rays	Electron	Ionization by primary and secondary electrons	Radioactive materials Electron accelerators
Proton or deuteron streams	H^1 or H^2 nucleus	Ejection of electron. Ionization by secondary electrons	Cyclotron
Fast neutrons	Neutron	Ejection of nuclei (H^1). Ionization by proton and tertiary electrons	Atomic pile
Slow neutrons	Neutron	γ-Ray from nuclear reaction. β and γ-ray production from new isotope	Atomic pile
X- and γ-rays	High energy electromagnetic radiation	Pair production. Compton effect. Photoelectric effect. Secondary electrons	X-ray generators Radioactive materials

by Lea (21), by several ionizations in or very close to the affected chromosomes. Visible breaks are not found in the highly coiled metaphase chromosomes (31), but they may become apparent at the next division. When a break occurs in a chromosome before duplication, both chromatids will show the same change at the same place. If only one chromatid is affected, it is assumed that the injury occurred after duplication and some estimates of the time of duplication have been based on this assumption. A broken end is usually left in such a condition that it readily joins with another broken end. It is believed that, in a majority of cases, the two ends reunite and reform the original chromosome. Such restitution (as it is called) may occur without any detectable damage remaining. Some broken chromosomes, however, are not restituted in this way. A broken end may link up with other broken ends of the same or a different chromatid or with fragments of chromatids. Practically every possible linkage of this sort may be found and some drastic rearrangements are possible (21). Most large permanent structural changes are lethal or result in a less viable cell; changes not involving loss of much genetic material are not usually lethal.

Susceptibility to damage by ionizing radiation varies throughout the mitotic cycle. Some data (31) indicate that susceptibility to chromosome breakage is greatest in metaphase when the chromosomes are in their most condensed forms, though these breaks do not become apparent until the next mitotic cycle.

Another important type of radiation effect is the production of mutations that may arise without any visible change in nucleus or chromosomes. Lea (21) has presented arguments based on the type and number of genetic changes found with different kinds of radiation, dose rate, etc., to show that, while a chromosome break requires several ionizations at the site of the break, a mutation may be produced by a single ionization.

The basis of these genetic and chromosomal changes is obscure partly because of lack of knowledge of genes and chromosomes. However some insight into the likely mechanism comes from the study of the effects of radiation on simpler aqueous systems (2, 21). A striking feature of radiochemical reactions in dilute aqueous solution is that the number of molecules of solute changed is linearly related to the radiation dose over a very wide range of solute concentrations. Since, in dilute solution, most of the radiation is absorbed by the solvent, this linear relation led to the concept of "activation" of water molecules which then passed on their energy to the solute (2). It is now generally agreed (1, 11) that this "activation" involves the production of hydroxyl radicals and hydrogen atoms according to the following series of reactions:

$$H_2O + h\nu \longrightarrow H_2O^+ + e^-$$

$$H_2O^+ + aq \longrightarrow H^+ aq + OH$$

$$e^- + H_2O + aq \longrightarrow OH^- aq + H$$

The over-all primary decomposition may be represented by

$$H_2O + h\nu \longrightarrow OH + H\,.$$

Exceptions to the simple relation between dose and change in the solute are found in some circumstances. At very low solute concentrations, possibly because of reactions of the radicals not involving the solute, the number of solute molecules altered by a given dose falls off with lowering concentration. The addition to the solution of another substance capable of reacting with the radicals reduces the amount of the original solute that is altered by a given dose. This "protective" effect is due to competition of the added substance with the original solute for the available radicals. When the products of the reaction are also capable of reacting with the radicals, they exert a protective effect on the original material. Under these circumstances, the linear relation between amount of solute changed and dose no longer holds. Lea and others (13, 21) have shown that the concentration of the original substance decreases exponentially with dose. This relation is the usual type found with complex organic compounds, enzymes, etc. (12).

The biochemical approach to the elucidation of the effects of ionizing radiation on genetic material has usually taken the form of studying *in vitro* irradiation of substances obtained from normal cells. The best known chromosomal constituents are desoxyribonucleic acid and the histones and protamines (15). Of these the nucleic acid is the best characterized. Also nucleic acid has been involved in the "sticky" chromosome phenomenon and mitotic inhibition (18, 23). Some extensive investigations therefore have been made of the effect of X- and γ-rays on this nucleic acid (5, 7, 22, 32, 33).

Sodium thymonucleate from calf thymus nucleoprotein has usually been chosen for these studies because it may be readily prepared. When isolated by mild methods, this substance is fibrous and highly polymerized. Reflecting this physical state is the high viscosity of dilute solutions. Since this viscosity has been found sensitive to radiation and may be conveniently measured, the effect of radiation has usually been assessed viscometrically. However, thymonucleate solutions deviate greatly from Newtonian behavior, actually forming weak gels at relatively low concentrations, and since different methods of measuring viscosity have been used by different investigators, the comparison of their results is difficult.

Irradiation of thymonucleate solutions results in a reduction of solution viscosity. Sparrow and Rosenfeld (32) reported an exponential decrease in the viscosity of 0.2–0.4% solutions of thymonucleate and nucleohistone following X-irradiation. A longer series of experiments were made by Taylor, Greenstein, and Hollaender (33). Solution viscosity was reduced by X-irradiation and a further slow decrease in viscosity that persisted for some time after the cessation of irradiation was noted. A number of tests indicated that only those properties connected with particle size were altered by X-rays. It was concluded that the nucleate molecules had been fragmented as a result of the irradiation.

Butler (5) attempted to test whether X- and γ-rays act directly upon thymonucleate in solution or indirectly through active radicals produced in the solvent. In this study most of the criteria required to demonstrate that

the action was indirect were satisfied. In irradiation of dilute nucleate solutions, the relation between total dose and viscosity, as log per cent of the control specific viscosity, is apparently linear (Fig. 1). This relation is to be expected if the products of the first attack of the active radicals on thymonucleate are also susceptible and if, as is approximately the case, the specific viscosity is proportional to the concentration of unaffected nucleate.

Glucose and methanol, when added to the thymonucleate solution, were found to be efficient protective agents. Thymonucleate, degraded by irradiation so that its viscosity increment was very low, when present in equal weight concentration, reduced the effect of γ-radiation on the tested thymonucleate by approximately one half. This proportion demonstrated that degraded thymonucleate has the same affinity for active radicals as undegraded nucleate.

The importance of water in the degradation of thymonucleate by X-rays is shown by the lack of effect, as tested by subsequent viscosity measurements, of irradiating dry nucleate, frozen nucleate solution, and nucleate dissolved in ethylene glycol (5, 22, 33). These experiments provide further evidence that the action of X-rays on nucleate is indirect and mediated through radicals produced in the water.

A number of groups of workers (6, 8, 22, 26, 29) have sought to identify the component of irradiated water that was responsible for the effect of X- and γ-rays on sodium thymonucleate. X- and γ-irradiated water contains, as the principal active reagents, hydrogen atoms and hydroxyl radicals but also smaller amounts of hydrogen peroxide, hydroperoxyl radicals (HO_2) and

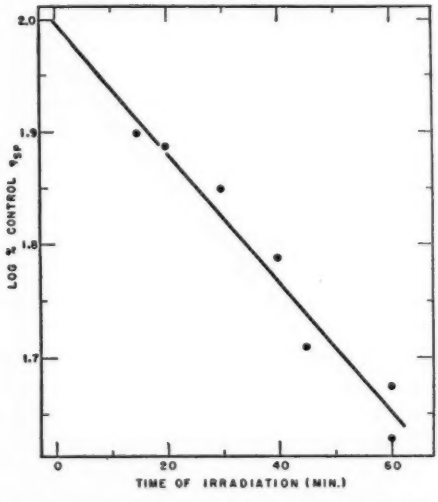


FIG. 1. The relation between viscosity and total dose of radiation. Solution (0.05%) of thymonucleate, Batch XXI, in 0.02 *M* sodium chloride irradiated with Co^{60} γ-rays at 1.94 r. per sec. (From Butler (5).)

oxygen atoms, arising from the action of the primary radicals with themselves and with dissolved oxygen. Several methods have been suggested for the identification of the component responsible for the effect of irradiation:

1. It may be possible to detect the evolution of either hydrogen or oxygen from the solution. If, for example, hydrogen atoms were reacting with nucleate to a greater extent than were hydroxyl radicals, the excess of the latter would form oxygen. However, when a sensitive oxidation–reduction indicator was used to test for the production of oxygen, none could be detected in a thymonucleate solution after an X-ray dosage of 40,000 roentgens (29). This result argues against a predominating effect of hydrogen atoms. No tests were made for the production of hydrogen.

2. The effect of hydrogen peroxide, which exists in small amounts in irradiated oxygen containing solutions, may be tested directly. Concentrations of hydrogen peroxide up to 0.15 *M* caused only a slow decrease in viscosity at room temperature (29, 33). The action of hydrogen peroxide then is entirely insufficient to account for the effect of X-irradiation of nucleate solutions since this amount is several orders higher than the peroxide content of irradiated solutions (3).

3. The effect of hydrogen atoms might be studied directly by treatment of solutions of thymonucleate with hydrogen and an active palladium black catalyst. When this method was tried (29), no change in viscosity resulted. Although the hydrogen molecule is considered to be dissociated at the palladium surface, this system does not, of course, reproduce the distribution of hydrogen atoms in an irradiated solution. Failure of hydrogen and palladium to reduce the viscosity of thymonucleate solutions does not, therefore, provide conclusive evidence against the reactivity of hydrogen atoms in irradiated solutions.

4. The effect of hydroxyl radicals may be tested directly. These radicals may be produced conveniently in neutral aqueous solutions by the interaction of hydrogen peroxide and ferrous ions (36) or by the photolysis of hydrogen peroxide with ultraviolet light of wave lengths shorter than about 370 mμ (34). Hydroxyl radicals produced in these ways in thymonucleate solutions proved effective in reducing solution viscosity and in many respects simulated the effects of X-irradiations (8, 22, 29). Neither hydrogen peroxide nor ferrous ions separately, nor irradiation with ultraviolet light in the absence of hydrogen peroxide, decreased viscosity relative to the control.

Fig. 2 shows the effect of irradiating with ultraviolet, solutions of sodium thymonucleate containing hydrogen peroxide (29). These results are typical. The relation between the viscosity, as log per cent of the control specific viscosity, and the length of the irradiation period is approximately linear. The same relation was found by Butler (5) when γ-irradiating a thymonucleate solution of similar concentration and electrolyte content (Fig. 1).

Fig. 3 shows the effect of varying nucleate concentration (29) on the rate at which the viscosity is reduced by peroxide and ultraviolet irradiation. The

rate is approximately constant at nucleate concentrations over about 0.04% and below this concentration falls off sharply. A falling off in the rate at about this concentration has been found with X-irradiation of various other organic substances (12, 21).

A study of the protective effect of glucose on the viscosity of nucleate solutions containing hydrogen peroxide and irradiated with ultraviolet

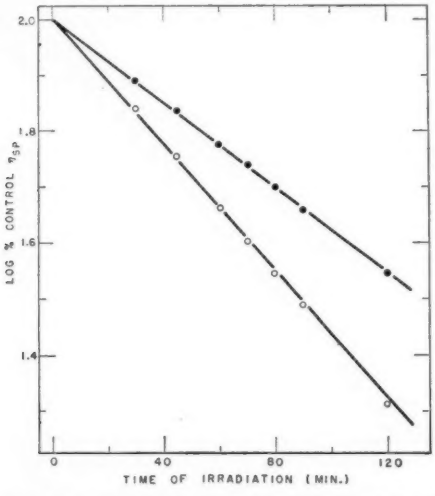


FIG. 2. The relation between viscosity and irradiation period. Solution (0.06%) of thymonucleate, Batch XXI, in 0.02 *M* sodium chloride containing 0.1 *M* hydrogen peroxide irradiated with ultraviolet light. ● Experiment 1; ○ Experiment 2. Light intensity less in Expt. 1.

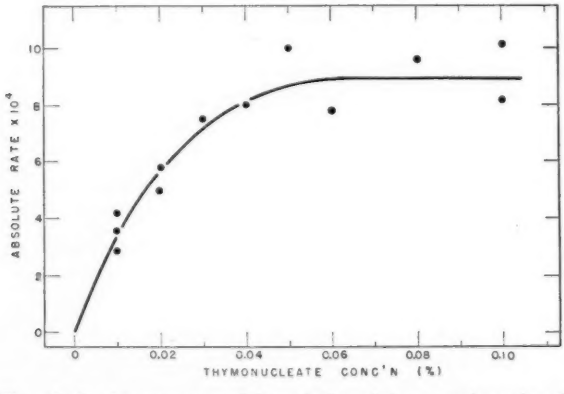


FIG. 3. The relation between rate of degradation of thymonucleate (= 50% decrease in viscosity × nucleate concentration/irradiation period) and nucleate concentration. Solution of thymonucleate, Batch XIII, in 0.02 *M* sodium chloride containing 0.1 *M* hydrogen peroxide irradiated with ultraviolet light.

showed (29) that only about 50% of the possible viscosity reduction of thymonucleate solutions was subject to competition (Fig. 4). A similar effect was found with methanol. (These results differ markedly from those obtained with the γ-irradiation of nucleate solutions containing these protective agents. In the latter study (5) smaller amounts of glucose or methanol gave protection approaching 100%.) When, however, thymonucleate, previously degraded with peroxide and ultraviolet light to reduce its specific viscosity to about 4% of its initial value, was used as the protective agent, no limit to its protective action was found (Fig. 4). Moreover at equal weight concentrations, the presence of the degraded nucleate reduced the effect on normal nucleate by about 50% (29). Butler (5) found the same effect with γ-irradiation. Such a result would be expected if the two forms of thymonucleate competed for active radicals on equal terms. This competition would also lead to an exponential relationship between viscosity loss and irradiation time which was the relation actually found (Fig. 2).

The anomalous behavior of glucose and methanol may be explained on the basis of absorption of hydrogen peroxide by thymonucleate. Hydroxyl radicals from this absorbed peroxide would attack the nucleate in preference to the added protective agents (unless the latter also absorbed peroxide). It would be expected that normal and degraded nucleate would bind peroxide similarly.

The number of hydroxyl radicals required for a 50% reduction in nucleate solution viscosity has been estimated for γ-rays by Butler (5) as about 60 μM. of radicals per gram of nucleate. When hydrogen peroxide and ultraviolet light were used to degrade thymonucleate, a maximum value of 250 μM. of hydroxyl radicals per gram of nucleate was calculated for the same viscosity decrease (29). In view of the various uncertainties involved, these estimates were considered to be in good agreement.

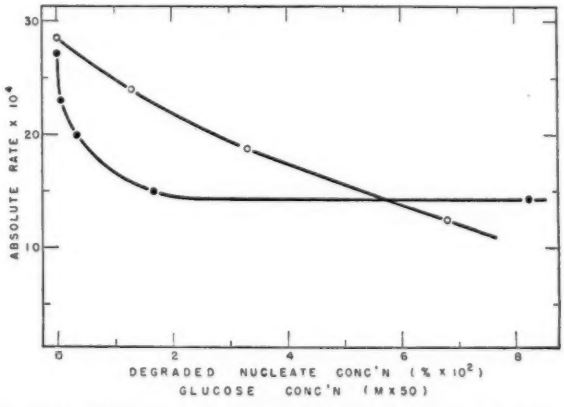


FIG. 4. Protective effect of glucose, ●, and of degraded nucleate, ○, on the degradation of thymonucleate, Batch XIII, in 0.02 *M* sodium chloride containing 0.1 *M* H_2O_2 irradiated with ultraviolet light.

5. If the hydroperoxyl radicals (HO_2) were the effective agents, there should be fewer of these in oxygen-free solutions since they are formed by the reduction of oxygen by hydrogen atoms. However, no difference in viscosity reduction was found when thymonucleate was irradiated in the presence of air or in the presence of nitrogen after careful removal of oxygen (29).

A progressive decrease in the viscosity of X-irradiated thymonucleate solutions that continued for some time after the cessation of irradiation was first noted by Taylor, Greenstein, and Hollaender (33). Others (5, 6) have confirmed the existence of this "aftereffect". The effect appears to be more marked with some preparations of thymonucleate than with others (though some of the differences may arise from the varied ways of measuring viscosity). Taylor *et al.* (33) reported that eight hours after stopping irradiation, the viscosity had approached a constant value at about 60% of that found immediately after irradiation. On the other hand, only a slight fall, ending in less than an hour, was noted by G. C. Butler (5). J. A. V. Butler and B. E. Conway (6) found that the viscosity drop was to about 60% of that found immediately after irradiation but that 20–30 hr. were required to reach this constant value. With dilute (0.06%) nucleate solutions, with and without added electrolyte, the viscosity decrease was found (28) to be less than 10% and to be complete in less than two hours. In all these studies, the solutions were exposed to, and probably saturated with, air. Butler and Conway (6) have reported that the "aftereffect" was much less in the absence of oxygen and suggested that it is due to the formation and subsequent slow breakdown of a compound formed by interaction of nucleate and hydroperoxyl radicals. Their nucleate preparation, however, was unique in being sensitive to small amounts of hydrogen peroxide and it is not clear that this property could not be responsible for their results. The "aftereffect" is not found following ultraviolet irradiation of thymonucleate solutions containing hydrogen peroxide (8, 29), though such solutions probably contain hydroperoxyl radicals arising from the reaction of hydrogen peroxide and hydroxyl radicals (1). However, the postulated unstable addition compound may have been decomposed immediately by the ultraviolet irradiation.

Weiss and his colleagues (24, 25, 26) have investigated the chemical changes resulting from the X-irradiation of sodium thymonucleate, nucleotides, and nitrogenous bases. Using X-ray doses of about two to four million roentgens on 2% sodium thymonucleate solutions, they demonstrated the formation of ammonia, dialyzable phosphate, inorganic phosphate, purines, and nucleosides, and an increase in titratable acid groups and amino nitrogen. They later reported detecting small amounts of ammonia and inorganic phosphate in dilute solutions (0.05%) of sodium thymonucleate after doses of only 30–150 thousand roentgens. The yield of ammonia was found to be increased in the presence of oxygen and decreased in hydrogen. These results indicated that the ammonia production was due to the oxidative radicals OH and HO_2. (Hydrogen atoms react with molecular oxygen to form HO_2 and in the presence of molecular hydrogen, hydroxyl radicals will be removed to form water.)

The formation of inorganic phosphate was enhanced when thymonucleate was irradiated in the presence of hydrogen. These results were interpreted as indicating that hydrogen atoms were more effective than hydroxyl radicals in causing dephosphorylation. There was also evidence of the formation of labile compounds which later yielded inorganic phosphate and it was suggested that the "aftereffect" might be related to the breakdown of these compounds.

The effects of X- and γ-rays and chemically produced hydroxyl radicals on sodium thymonucleate are sufficiently similar to suggest similar modes of action. It seems certain that hydroxyl radicals are responsible for part, at least, of the effect of ionizing radiations in sodium thymonucleate *in vitro*. From chemical studies, Scholes and Weiss (26) have concluded that the effect of irradiation on the nitrogenous bases of thymonucleate is primarily oxidative. The lack of effect of hydrogen, in the presence of palladium black, has been taken (29) to indicate that degradation of thymonucleate is not due to hydrogen atoms. However, Scholes and Weiss (26) concluded that their evidence points to an effect of hydrogen atoms. It is clear that radiation-produced hydrogen peroxide and hydroperoxyl radicals are not responsible for the main effect of ionizing radiations on the viscosity of thymonucleate solutions.

The decrease in the viscosity of irradiated thymonucleate solutions suggests that the gross effect is a reduction in particle size. Other lines of evidence lead to the same conclusion. Conway, Gilbert, and Butler (9) have measured by sedimentation and diffusion methods the molecular weights of sodium thymonucleate before and after 40,000 r. of X-rays. The average molecular weight fell as a result of the radiation from 1.4 million to 530,000. By light-scattering methods, Smith and Sheffer (30) have shown that treatment with hydrogen peroxide and ultraviolet reduced the molecular weight of their sample of thymonucleate from 4.3 million to 740,000. Electron micrographs of thymonucleate degraded both by X-rays (33) and by peroxide and ultraviolet light (29) showed a similar breakup of the long strands characteristic of electron micrographs of normal sodium thymonucleate (27).

The significance of these findings in the problem of the biological effects of ionizing radiations is not yet clear. Doses of 10,000 r. or more are necessary for detectable changes *in vitro* while biological effects may be produced by doses of only 100 r. or even less. Weiss (35) has recently suggested a mechanism which would provide the multiplication factor required to understand the high sensitivity of biological systems. Since he and his colleagues have found that X-rays cause the liberation of ammonia and inorganic phosphate in thymonucleate solutions, he suggests that the nitrogenous base and the sugar components are most susceptible to attack. Unstable phosphate esters may be produced which undergo hydrolysis with release of inorganic phosphate and rupture of the nucleic acid fiber. This elimination of amino and hydroxyl groups would result in a loss of hydrogen bonding which would

further tend to disorganize the nucleic acid structure. These suggestions, though still somewhat vague, are a step in interpreting the biological results in terms of *in vitro* findings.

For the production of gene mutations, it seems sufficient to suggest that alterations are produced either by the reaction of radicals with the gene or by an ionization within it.

References

1. Allen, A. O. J. Phys. & Colloid Chem. 52 : 479. 1948.
2. Allsopp, C. B. Brit. J. Radiology, Suppl. 1 : 43. 1947.
3. Bonet-Maury, P. and Lefort, M. Compt. rend. acad. sci. 226 : 1363. 1948.
4. Brues, A. M. and Reitz, L. Ann. N.Y. Acad. Sci. 51 : 1497. 1951.
5. Butler, G. C. Can. J. Research, B, 27 : 972. 1949.
6. Butler, J. A. V. and Conway, B. E. J. Chem. Soc. 3418. 1950.
7. Butler, J. A. V., Gilbert, L. A., and Smith, K. A. Nature, 165 : 714. 1950.
8. Butler, J. A. V. and Smith, K. A. Nature, 165 : 847. 1950.
9. Conway, B. E., Gilbert, L., and Butler, J. A. V. J. Chem. Soc. 3421. 1950.
10. Curtis, H. J. Advances in Biol. and Med. Phys. 2 : 1. 1951.
11. Dainton, F. S. Ann. Rept. Chem. Soc. 45 : 5. 1949.
12. Dale, W. M., Gray, L. H., and Meredith, W. J. Trans. Roy. Soc. (London), A, 242 : 33. 1949.
13. Dale, W. M., Meredith, W. J., and Tweedie, M. C. K. Nature, 151 : 280. 1943.
14. Darlington, C. D. Nature, 149 : 66. 1942.
15. Davidson, J. N. The biochemistry of the nucleic acids. Methuen & Co. Ltd., London. 1950.
16. Gray, L. H. Progress in Biophys. 2 : 240. 1951.
17. Hevesy, G. Revs. Modern Phys. 17 : 102. 1945.
18. Hevesy, G. Nature, 163 : 869. 1949.
19. Koller, P. C. Nature, 151 : 244. 1943.
20. Koller, P. C. Discovery, 10 : 241. 1949.
21. Lea, D. E. Actions of radiations on living cells. Cambridge University Press, London. 1946.
22. Limperos, G. and Mosher, W. A. Am. J. Roentgenol. Radium Therapy, 63 : 681. 1950.
23. Mitchell, J. S. Brit. J. Radiology, 16 : 339. 1943.
24. Scholes, G., Stein, G., and Weiss, J. Nature, 164 : 709. 1949.
25. Scholes, G. and Weiss, J. Nature, 166 : 640. 1950.
26. Scholes, G. and Weiss, J. Exptl. Cell. Research, Suppl. 2, 219. 1952.
27. Scott, J. F. Biochim. et Biophys. Acta 2 : 1. 1948.
28. Smith, D. B. Unpublished data. 1952.
29. Smith, D. B. and Butler, G. C. J. Am. Chem. Soc. 73 : 258. 1951.
30. Smith, D. B. and Sheffer, H. Can. J. Research, B, 28 : 96. 1950.
31. Sparrow, A. H. Ann. N.Y. Acad. Sci. 51 : 1508. 1951.
32. Sparrow, A. H. and Rosenfeld, F. M. Science, 104 : 245. 1946.
33. Taylor, B., Greenstein, J. P., and Hollaender, A. Arch. Biochem. 16 : 19. 1948.
34. Urey, H. C., Dawsey, L. H., and Rice, F. O. J. Am. Chem. Soc. 51 : 1371. 1929.
35. Weiss, J. Nature, 169 : 460. 1952.
36. Weiss, J. and Humphrey, C. W. Nature, 163 : 691. 1949.

DISCUSSION

Q.—**Dr. S. G. A. Alivisatos:** What is meant by the term radiomimetic compound and how do these substances act?

A.—**Dr. Smith:** Radiomimetic compounds have been defined as substances capable of producing genetic changes and chromosome breakages in resting

cells. The broken chromosomes should be capable of rejoining so as to produce deletions, rearrangements, etc. They are thus different from other mutagenic substances, mitotic poisons and other materials affecting the mitotic cycle in other ways. The most potent radiomimetic compounds are the nitrogen and sulphur mustards. Others are ethylene oxide, ethylene imines, alkyl peroxides, formalin, and urethane. The similarities between the genetic effects of these substances and X-irradiation are superficially very striking. Closer analysis, however, reveals many differences. There is as yet no satisfactory hypothesis to account for their action and it is quite likely that the various groups of compounds act in different ways.

Q.—**Dr. Zamenhof:** Weiss postulates that ammonia is produced in tissues on exposure to heavy doses of radiation while this does not occur with small doses. May there be a threshold dose? Also has Weiss not suggested that mutants may be produced by the action of ammonia produced in this way?

A.—**Dr. Smith:** I do not recall Weiss's postulate. Weiss found that less ammonia is produced by smaller doses of radiation, but his published data give no indication that there is a threshold dose. His graphs, when extrapolated, indicate a zero yield of ammonia at zero dose of radiation. Deamination appears to proceed readily. There was some indication that the liberation of inorganic phosphate occurs only after an initial reaction of some sort.

Weiss thought also that since ammonia has been shown to increase the rate of mutation, the mutagenic effect of radiation might be due, in part, to the action of radiochemically produced ammonia.

ROUND TABLE DISCUSSION HELD IN EVENING

CHAIRMAN: DR. O. F. DENSTEDT

Speakers' Panel: Drs. Butler, Elwyn, Stevens, Zamenhof, Graham, Smith.

The Chairman opened the meeting by presenting to the panel members the question: What are some of the salient questions concerning the nucleoproteins which require elucidation at this time, and what developments may be expected within the next few years?

Dr. Zamenhof *(Columbia University)*: Our knowledge of the protein moiety of the nucleoprotein molecule is rudimentary. For this reason I prefer to answer the first question only with reference to the nucleic acid component and especially the DNA. To the chemist, the most pressing questions and the ones which are likely to be partially answered within the next 10 years are:

(*a*) What active groups and chemical structures are essential to biological activity?

(*b*) What is the nature of the changes in the chemical structure of the DNA accompanying the production of mutations?

Among the questions unlikely to be solved in the near future are:

(*c*) What is the chemical mechanism of the "true to original" duplication of DNA?

(*d*) By what mechanisms is the DNA connected with the chemical processes of the cell?

Dr. Butler *(University of Toronto)* added that in his opinion the most important problem for biochemists is the elucidation of the relation of the chemical structure of the nucleic acids to their biological activity. Before much progress can be made in such a study it will be necessary to devise methods of fractionating nucleates in order to obtain homogeneous samples. At the moment this technical problem appears to be almost impossible of solution.

The meeting was then thrown open for discussion of questions which had been submitted by various members of the audience during the day session and directed to members of the panel. Members of the audience also were invited to contribute to the general discussion.

Q.—Is there any information as to how bacteria produce the transforming principle?

A.—**Dr. Zamenhof:** My view (which is merely speculative) is that in most cases a particle of the original transforming principle penetrates the nucleus, finds a specific locus on a specific chromosome (if there are chromosomes in bacteria), attaches itself to the existing protein or produces its own protein, and from then on behaves like any other bacterial gene. In other words, it self-duplicates together with the others thus producing more of the same transforming principle.

Q.—Is anything known about the biochemical mechanism of the viral interference effect?

A.—**Dr. Graham:** With reference to the coliphages one common example of viral interference is as follows. If the host is infected simultaneously with related phages T2 and T4 both will grow in the cell and produce progeny. If the cell is first infected with T2, and T4 is added later, the yield of T4 decreases as the interval is increased until exclusion of T4 is almost complete with a five minute interval.

One possible mechanism for this exclusion is an enzymatic breakdown of the second virus induced by the first infection. The DNA of the second virus is, in fact, rapidly broken down to acid soluble products to the extent of 50% when there is an interval of five minutes between infections. However, it now seems very doubtful whether this breakdown is the means whereby the second virus is excluded. It is not a general mechanism for exclusion in any case since the superinfection breakdown is not observed in some cases of interference between pairs of unlike phages.

Referring to interference between the even numbered phages another aspect of the problem is that the DNA phosphorus of the second virus is also excluded from the progeny. There is a very close correlation between exclusion of this phosphorus, exclusion of the genetic markers of the second virus and the breakdown of the second virus DNA as the interval between infections increases. If we admit that the breakdown correlation is probably fortuitous we still have to consider the relationship of phosphorus transfer and that of genetic markers. According to one current concept the transfer of phosphorus is unassociated with genetic transfer. If this is so it is exceedingly difficult at present to postulate a biochemical mechanism for interference. On the other hand if later work does not show an association of transferred phosphorus and genetic units it will at least provide a starting-point for experimental attack.

Taking another example of interference, if one infects a cell with T7 and several minutes later adds T2, only T2 will be found in the viral yield. Although growth of T7 had already begun it is completely suppressed by the second virus and only T2 grows. While many examples of this type of interference are known, any attempt to provide a mechanism is pure speculation.

Q.—What is the chemical interpretation of a mutation?

A.—**Dr. Zamenhof:** As mentioned previously this is one of the most important problems, and as yet is unsolved. The change in specificity without inactivation might perhaps result from the removal or addition of nitrogenous bases, from a change in their order of arrangement, from a modification of the spatial configuration or hydrogen-binding, or other types of alteration. All these suggestions, of course, are purely speculative.

Q.—How dangerous are ionizing radiations and how do they produce the alteration of chromosomes?

A.—**Dr. Smith** *(National Research Council, Ottawa)*: High dosages of penetrating ionizing radiation may cause death after a lag period ranging from a few hours to several weeks, depending on the intensity of the dosage. The illness known as 'radiation sickness' may vary from slight nausea to severe illness resulting in death. The blood-forming tissues are the first to be noticeably affected. Radiation can "burn" the skin and may produce cancer. Genetic effects may be produced by a single or repeated exposure to radiation. Most of the genetic effects may not become detectable until far in the future. The degree to which they may be detrimental is as yet difficult to assess.

It has been suggested that the chromosomes are damaged by ionizing radiations either by direct ionization leading to chemical changes in the chromosomal substances, or by the production of free radicals or other substances which then may react with the chromosomes. However, as Dr. Zamenhof has remarked, the nature of the changes is still unknown.

The Chairman asked Dr. L. E. Hokin whether he would comment on the role of the nucleic acids in the synthesis of proteins.

A.—**Dr. Hokin** (*Montreal General Hospital Research Institute*): Many believe that nucleic acids are the genetic determinants in chromosomes and viruses. Certainly, the pneumococcus transforming factor affords strong evidence that nucleic acids may direct genetic specificity.

There is also indirect evidence that nucleic acids are concerned in the synthesis of proteins. It has been known since the time of Miescher that nucleic acids reversibly combine with proteins. It has recently become clear that nucleic acids are complex in structure, a circumstance which could account for a variety of specific nucleic acid polymers. The two properties of nucleic acids, namely, the ability to combine with proteins and the specificity of structure, enable us to construct a simple hypothesis to explain numerous phenomena in which nucleic acids are involved. Thus the nucleic acids, both DNA and RNA, may function by providing a framework for the organization of "synthesizing enzymes" (peptidases, etc.) into specific patterns. These enzymes, attached to the various types of nucleic acid framework, might thus be capable of catalyzing the synthesis of specific polymers such as nucleic acids, proteins (enzymes), and polysaccharides according to the specific patterns. Such a scheme postulates a simple mechanism whereby nucleic acids may direct the synthesis of enzymes in the cell and whereby genes, viruses, and the pneumococcus transforming factor may direct their own specific synthesis as well as that of other polymers.

Q.—Are the nucleoproteins antigenic?

A.—**Dr. Zamenhof:** The nucleoproteins are antigens. The prevailing opinion is that the antigenicity is due to the protein moiety, as the nucleic acids themselves appear to be immunologically inactive.

Q.—What is the evidence that radiation effects are produced through a free radical mechanism?

A.—**Dr. Smith:** The evidence comes from a study of the radiochemical changes in simpler aqueous systems. The results of these studies are best interpreted on the basis of free radical formation in the solvent.

Q.—How useful is the bacteriophage system for screening for antibiotics against animal viruses?

A.—**Dr. Graham:** Bacteriophage systems provide a very rapid and precise means of testing a large number of compounds for antiviral activity. Any that looks promising can then be tested against animal virus systems. However, one might test the antiphage compound against the wrong animal viruses and find no activity or pass over possible animal virus inhibitors that show no antiphage activity. In my opinion this type of "bottle-off-the-shelf" experimentation is seldom worth the effort. For some years past it has been apparent that virus reproduction is so intimately connected with the fundamental processes of cell growth that the more thoughtful academic approach to the problem is more likely to provide an antiviral therapy than the hit-or-miss method.

Q.—It would appear that irradiation of blood plasma with ultraviolet light is not very satisfactory as a means of sterilization against the virus of homologous serum jaundice. What is the relative lability of the serum proteins and the viruses to irradiation with ultraviolet? Is it likely that the serum proteins would be damaged along with the virus?

A.—**Dr. Smith:** I am not sure of the answer to the first question, but it is safe to say that the serum proteins would be affected by a strong ultraviolet irradiation. There would likely be a wave-length effect since the absorption maximum of nucleic acid is about 2600 Å, while that of the aromatic amino acids is in the range of 2700-2800 Å.

CANADIAN JOURNAL OF MEDICAL SCIENCES

Notes to Contributors

GENERAL: Manuscripts should be typewritten, double spaced, and the **original and one extra copy** submitted. Style, arrangement, spelling, and abbreviations should conform to the usage of this Journal. Names of all simple compounds, rather than their formulas, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed. Manuscripts should be carefully checked before being submitted, to reduce the need for changes after the type has been set. If authors require changes to be made after the type is set, they will be charged for changes that are considered to be excessive. **All pages, whether text, figures, or tables, should be numbered.**

ABSTRACT: **An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required.**

ILLUSTRATIONS:

(i) *Line Drawings:* All lines should be of sufficient thickness to reproduce well. Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper **ruled in blue only**; any co-ordinate lines that are to appear in the reproduction should be ruled in black ink. Paper ruled in **green, yellow, or red should not be used** unless it is desired to have all the co-ordinate lines show. Lettering and numerals should be neatly done in India ink preferably with a stencil (**do not use typewriting**) and be of such size that they will be legible and not less than one millimeter in height when reproduced in a cut three inches wide. All experimental points should be carefully drawn with instruments. Illustrations need not be more than two or three times the size of the desired reproduction, but the ratio of height to width should conform with that of the type page. **The original drawings and one set of small but clear photographic copies are to be submitted.**

(ii) *Photographs:* Prints should be made on glossy paper, with strong contrasts; they should be trimmed to remove all extraneous material so that essential features only are shown. Photographs should be submitted **in duplicate**; if they are to be reproduced in groups, one set should be so arranged and mounted on cardboard with rubber cement; the duplicate set should be unmounted.

(iii) *General:* **The author's name, title of paper, and figure number should be written in the lower left hand corner (outside the illustration proper) of the sheets on which the illustrations appear.** Captions should not be written on the illustrations, but typed together at the end of the manuscript. All figures (including each figure of the plates) should be numbered consecutively from 1 up (arabic numerals). **Each figure should be referred to in the text.** If authors desire to alter a cut, they will be charged for the new cut.

TABLES: Each table should be typed on a separate sheet. Titles should be given for all tables, which should be numbered in Roman numerals. Column heads should be brief and textual matter in tables confined to a minimum. **Each table should be referred to in the text.**

REFERENCES: **These should be listed alphabetically by authors' names, numbered in that order, and placed at the end of the paper.** The form of literature citation should be that used in this Journal. **Titles of papers** in the references cited should **not** be given. Initial page numbers only are required. **All citations should be checked with the original articles.** Each citation should be referred to in the text by means of the key number.

REPRINTS: A total of 50 reprints of each paper, without covers, are supplied free to the authors. Additional reprints will be supplied according to a prescribed schedule of charges. On request, covers can be supplied at cost.

Approximate charges for reprints may be calculated from the number of printed pages, obtained by multiplying by 0.6 the number of manuscript pages (double-spaced typewritten sheets, 8½ in. × 11 in.) and making allowance for space occupied by line drawings and halftones (not inserts). The cost per page is tabulated on the back of the reprint request form sent with the galley.

Contents